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(54) Title: NUCLEIC ACID ENCODING A NOVEL CHEMOTHERAPY-INDUCED PROTEIN, AND METHODS OF USE

(57) Abstract

An isolated nucleic acid molecule is provided which encodes a mammalian signal transducing protein (Killer) involved in the induction of apoptosis. The encoded protein comprises an amino-terminal extracellular domain, a hydrophobic transmembrane domain, and a carboxy-terminal death domain that can form protein-protein interactions with other death domain-containing proteins to transmit the signals required for apoptosis. The invention also provides the Killer protein and antibodies thereto. These biological molecules are useful as diagnostic and therapeutic agents for the identification, detection and regulation of complex signaling events leading to apoptotic death. The molecules may also be used to advantage for assessing the potential chemosensitivity of a targeted tumor cell. Additionally, these molecules may be used as research tools to facilitate the elucidation of the mechanistic action of the Killer proteins of the invention.

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NUCLEIC ACID ENCODING A NOVEL CHEMOTHERAPY-INDUCED PROTEIN, AND METHODS OF USE

This application claims priority to the following United States Provisional Applications, all of which are incorporated by reference herein: 60/052,305, filed July 11, 1997; 60/054,710, filed August 4, 1997; 60/060,473, filed September 30, 1997; 60/077,661, filed March 11, 1998; 60/077,628, filed March 11, 1998; and 60/077,526, filed March 11, 1998.

FIELD OF THE INVENTION

This invention relates to diagnosis and treatment of neoplastic diseases. More specifically, this invention provides novel nucleic acid molecules, proteins and antibodies useful for detection and/or regulation of neoplastic cellular changes.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application to more fully describe the state of the art to which this invention pertains. The disclosure of each such publication is incorporated by reference herein.

The p53 tumor suppressor protein is a nuclear phosphoprotein that functions in cell-cycle arrest, programmed cell death (apoptosis), inhibition of tumor growth, and preservation of genetic stability. It performs these functions through involvement in several biochemical pathways, including transcriptional activation, transcriptional suppression and inhibition of DNA replication. Loss of proper p53 function in cells is one step in the progression toward a neoplastic phenotype; more than 50% of human cancers have mutations in the p53 gene (see V.E. Velculescu & W.S. El-Deiry, Clin. Chem., 42:858-868, 1996; A.J. Levine, Cell 88: 321-331, 1997).

DNA binding and transcriptional activation are among the best-understood functions of p53. Nearly every tumor-derived p53 mutant has lost its ability to bind DNA and transcriptionally activate nearby genes. This observation suggests that these properties of p53 are critical to its role in the control of cell proliferation. The diverse nature of the genes that p53 transcriptionally regulates suggests that p53 may be involved in pathways of cell-cycle control, angiogenesis, DNA repair, differentiation, growth factor signaling and apoptosis.

Experimental evidence indicates that p53 activates a cell suicide pathway (apoptosis) after treatment with chemo- or radiotherapy. In some cell types, the cytotoxic action of anti-cancer agents may be mediated through p53-dependent apoptosis. p53 regulates a number of genes involved in apoptosis, including *bax*, *bcl-2*, insulin-like growth-factor binding protein-3 (*igf-bp3*), and *fas*. The genes for Bax and IGF-BP3 have been shown to contain p53-dependent, *cis*-acting, DNA-responsive elements.

Inactivation or mutation of p53 has been reported to enhance cell sensitivity to cytotoxic agents which induce DNA damage. However, the status (wild type or mutant) of p53 in tumor cells does not always correlate with chemosensitivity. Endogenous p53 status predicts *in vitro* chemosensitivity to the clinically useful agents Adriamycin, Carboplatinum, Cytoxan, and Etoposide in only a minority of cancer cell lines (ovarian and some Burkitt's lymphoma cells), but not in others (e.g., leukemia or lung cancer cells). Several recent studies have shown that many clinically useful drugs appear to be more effective in *in vitro* killing of tumor cell lines that contain wild-type p53. Furthermore, HPV16 E6-mediated degradation of p53 in a human lung cancer cell line does not render these cells more resistant to Adriamycin or Etoposide. By contrast,

human ovarian teratocarcinoma cells become more chemoresistant and display less death by apoptosis when the endogenous p53 protein is degraded by E6 expression. Additionally, mutations in the p53 gene do not correlate with radioresistance in a series of human squamous carcinoma cells. Finally, recent studies have shown decreased patient survival in patients who have aggressive tumors which have lost p53 following chemo- or radiotherapy (Velculescu, et. al., 1996, supra).

Certain cytokines interacting with their cognate receptors on the surface of target cells also trigger apoptosis. For example, Fas ligand (FasL) and tumor necrosis factor (TNF) are members of the TNF family of cytokines that trigger apoptosis in target cells through binding and trimerization of their respective cell surface receptors, Fas and TNF receptor 1 (TNFR1). Another TNF family cytokine is TRAIL, the cytotoxic ligand for the recently cloned DR4 receptor (Pan et al., Science 276: 111-113, 1997). Once trimerized, the death domains in the C-termini of the receptors form protein-protein interactions with the C-terminal death domains of intracellular death signal effector molecules, initiating a cascade of signals (involving activation of caspases and endonucleases) that ultimately cause apoptosis.

Wild-type p53 has been shown to upregulate production of Fas/APO-1 mRNA and protein, but as yet, no p53-responsive DNA binding site has been identified in the *fas* gene. Thus p53 has not been shown to directly regulate the transcription of a TNFR family member in an apoptosis pathway.

It is clear from the foregoing discussion that the relationship between p53 status and chemo- and radiosensitivity is not sufficiently understood to warrant its use in cancer therapy. As set forth above, p53 status in a tumor does not necessarily predict therapeutic outcome following such treatment. Moreover, gene replacement therapy with wild-type p53 may be

ineffective, and for some tumors counterproductive, as it is not as yet predictable which tumors would respond positively or negatively to such therapy. For these reasons, there is a need to identify downstream targets of p53 function in apoptotic signaling. Once identified, such p53 targets may be utilized or regulated in apoptosis-based therapeutic approaches to the elimination of tumor cells.

SUMMARY OF THE INVENTION

The present invention provides novel nucleic acid molecules that are themselves targets of p53 activity and whose encoded proteins are involved in modulation of cellular proliferation. According to one aspect of the present invention, an isolated nucleic acid molecule is provided that includes an open reading frame encoding a mammalian p53-inducible, apoptosis-mediating protein. The protein comprises an amino-terminal extracellular domain, a transmembrane domain, and a death domain that facilitates protein-protein interactions.

In a preferred embodiment of the invention, the nucleic acid molecule is of human origin, and comprises a sequence substantially the same as SEQ ID NO:1, or at least substantially the same in the death domain-encoding region as SEQ ID NO:1. The invention also comprises a nucleic acid having the sequence of SEQ ID NO:1. The encoded protein, which is referred to herein as "Killer," for reasons described below, preferably comprises a sequence substantially the same as SEQ ID NO:2, or at least substantially the same in the death domain of SEQ ID NO:2. A Killer protein encoded by SEQ ID NO:2 is another preferred embodiment of the invention.

In another aspect of the invention, oligonucleotides that specifically hybridize with selected portions of the nucleic acid molecules of the invention are provided.

In yet another aspect of the invention,

antibodies immunologically specific for the Killer proteins of the invention, or fragments thereof, are provided.

According to other aspects of the present invention, uses of the nucleic acid molecules of the invention, their encoded proteins, as well as antibodies to those proteins, for diagnostic and therapeutic purposes are provided. Specifically, methods are provided for assessing the induction of "*Killer*" expression following exposure of tumor cells to chemotherapeutic agents. In accordance with the present invention, *Killer* expression is assessed in conjunction with p53 status. These methods will facilitate identification of efficacious chemotherapeutic agents as measured by the induction of apoptosis in the targeted tumor cell.

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims. The terms "substantially the same," "percent similarity" and "percent identity (identical)" are defined in detail in the description set forth below.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5′ and 3′ directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote.

With respect to RNA molecules of the invention, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from

RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

With respect to antibodies of the invention, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest (e.g., "Killer"), but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to oligonucleotides, the term "specifically hybridizing" refers to the association between two single-stranded nucleotide molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded

DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The nucleic acids, proteins and antibodies of the present invention are useful as diagnostic and therapeutic agents for the detection and treatment of cancer and other proliferative diseases. They should also find utility as research tools and will facilitate the elucidation of the mechanistic action of the novel genetic and protein interactions involved in the control of chemotherapy induced cell death.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the nucleic acid sequence of *Killer*, a novel adriamycin-inducible transcript. The figure shows the encoding nucleotide (SEQ ID NO:1) and predicted protein translation (SEQ ID NO:2) of the *Killer* open reading frame. The death domain is enclosed in a box, and the putative transmembrane domain and signal peptide sequences are underlined by double and single lines, respectively. The termination codon is indicated by a star. *Killer* has a perfect Kozak sequence upstream of the initiating methionine 5'-CCGCCATGG-3'.

Figs. 2A and 2B show protein sequence homology between Killer and related proteins. Amino acid homology between *Killer* and its closest homolog DR4 (SEQ ID NO:3) is depicted in Fig. 2A. In the case of DR4, only the predicted mature protein sequence is shown (Pan et al., 1997, *supra*). Identity and similarity are indicated by a star and a colon, respectively. The pairwise alignment was carried out using the MacVector™ 6.0 Clustal W(1.4) program (Oxford Molecular Group). Fig. 2B indicates the homology in the death domain regions of Killer and other TNF receptor family members. A multiple sequence alignment algorithm was carried out using the MacVector™ 6.0 Clustal W(1.4) program (Oxford Molecular Group). A

predicted death domain consensus is shown below the alignment. The degree of greyness reflects the degree of conservation between different family members. The following amino acids were included within the death domain of each family member: Killer aa 324-393 (part of SEQ ID NO:2); DR4 aa 356-404 (part of SEQ ID NO:3), DR3 aa 346-392 (SEQ ID NO: 4), Fas/apo1 aa 228-277 (SEQ ID NO:5), TNFR-1 aa 330-380 (SEQ ID NO:6), and CAR 1 aa 315-333 (SEQ ID NO:7).

Figs. 3A, 3B and 3C show regulation of *Killer* gene expression by wild-type p53. Fig. 3A: Northern analysis of *Killer* expression (upper panels) was carried out using RNA derived from different human cancer cell lines (as indicated) either untreated (-) or 10 hrs after continuous treatment (+) with an apoptosis-inducing concentration 0.3 μg/ml) of doxorubicin (adriamycin, adria) or 500 Rads ionizing radiation. Fig. 3B: Northern analysis shows that *Killer* mRNA expression is increased in H460 lung cancer cells 10 hours after continuous treatment with 0.3 μg/ml doxorubicin (adriamycin), 5 μM Etoposide or a dose of 2,000 rads ionizing radiation at time zero. Fig. 3C: Northern analysis showing induction of *Killer* mRNA expression in mutant p53-expressing colon, ovarian or breast cancer cells 12 hours after infection by either Ad-LacZ or Ad-p53. An ethidium stain of the RNA (lower panels) indicates equivalent RNA loading (10 μg) of the lanes in each experiment, and the p53 status of each cell line is shown below the ethidium stains (Figs 3A, 3C).

Fig. 4 is a graph illustrating that expression of the death domain in *E. coli* is toxic. Bacteria were transformed with pGEX-GD vector encoding the death domain of Killer in the presence (open squares, open circles) or absence (closed squares, closed circles) of glucose. Induction of death domain expression by IPTG resulted in a dramatic decrease in absorbance which was unaffected by the presence or absence of glucose ruling out a

catabolite repression effect.

Fig. 5 shows that *Killer* is not induced in p53-dependent G1 arrest. Fig 5A: to induce p53-dependent cell cycle arrest in the absence of DNA damage, GM cells carrying a dexamethasone-inducible wild-type p53 exogenous transgene were incubated in the absence (lane 1) or presence (lanes 2-4) of dexamethasone. After incubation for increasing periods of time (as indicated) total RNA was isolated and Northern blot analysis, using either the human *Killer* (upper panel) or the human *p21* (middle panel) cDNA as probes, was performed as described in Materials and Methods. An ethidium bromide stain of the RNA is shown (lower panel) to document equivalent loading of the gel. Fig. 5B: to induce a DNA damage/p53-dependent cell cycle arrest, WI38 cells were exposed to either 2 Gy or 10 Gy g-radiation. Total RNA was harvested at 10 hrs and Northern blotting performed as in A using either the *Killer* (upper panel) or the *p21* (middle panel) cDNA as a probe. An ethidium bromide stain of the RNA is shown (lower panel) to document equivalent loading of the gel.

Figs. 6A and 6B show expression of *Killer* in cells undergoing apoptosis following Ad-*p53* infection. Fig. 6A: expression of *Killer* is increased in GM cells undergoing apoptosis but not growth arrest. Northern blots of *Killer* mRNA expression in GM cells that were untreated (lane 1), treated with dexamethasone to induce a p53-dependent cell cycle arrest (lane 2), infected by Ad-LacZ as a control (lane 3) or infected by Ad-p53 (lane 4). An ethidium bromide stain is shown (below; 28S and 18S RNA indicated by arrows) to document equivalent loading of the RNA samples. Fig. 6B: p53-dependent upregulation of *Killer* mRNA expression occurs early in SKOV3 cells and continues for the next 48 hrs. Northern analysis of total RNA harvested at increasing time points (as indicated) following infection of SKOV3 cells by Ad-LacZ (lanes 2, 4, 6, 8, 10) or Ad-p53 (lanes 3, 5, 7,

9, 11) for *Killer* expression.

Figs. 7A and 7B show transcriptional control of TRAIL death receptor expression in p53-dependent apoptosis. The human ovarian carcinoma cell line SKOV3 (Fig. 7A) or the human glioblastoma cell line GM (Fig. 7B) were infected by Ad-LacZ (lanes 1,2) or Ad-p53 (lanes 3,4). Following infection for one hour, cells were incubated either in the absence ("-" as indicated; lanes 1,3) or in the presence ("+" as indicated; lanes 2,4) of 30 ng/ml Actinomycin D for an additional 20 hrs when total cellular RNA was harvested for northern blotting for expression of *Killer*, *p21*, *MDM2*, and *p53* mRNA (as indicated). An ethidium stain of the RNA is shown (lowest panels) to document equivalent loading of the gel.

Fig. 8 is a diagram of the physical map of the coding region of the human *Killer* gene. Exons are shown as black boxes; the number of the exon ("e") together with its size (in kb) are indicated above the boxes. The introns are shown as clear boxes and their respective sizes (in kb) are written within. The double headed arrows below represent the products of PCR reactions, shown together with their respective sizes. The translation initiation codon is depicted with a bent arrow, and the translation stop codon with a blunted arrow. The genomic position of the signal sequence (SS), the transmembrane domain (TM) and the death domain (DD) are indicated above the bar. The positions and the number of the extracellular Cysteine residues (C) are drawn above the bar. The gray boxes represent the position of clones obtained through subcloning P1-*Killer*, PstI digestion fragments (hybridizing to probes-A and B on Southern blots, shown above the boxes), into pBluescript.

Fig. 9 is the nucleotide sequence of a genomic clone of *Killer*, "*P1-Killer*" (SEQ ID NO:14). The putative CAT and TATA boxes are indicated, as is the putative start codon.

DETAILED DESCRIPTION OF THE INVENTION

As described above, p53 plays a pivotal and complex role in preventing malignant transformation. In some instances, p53 has been found to activate apoptosis after treatment of cells with chemotherapeutic agents or radiation. However, the relationship between p53 status and sensitivity or resistance to such treatments remains unclear, making it difficult to design or implement therapeutic strategies based on p53. Identification of downstream targets of p53 function in apoptotic signaling would help overcome this difficulty. One such target has now been identified in accordance with the present invention.

Using a subtractive hybridization technique, the inventors have identified a unique cDNA clone corresponding to a gene whose expression is upregulated in Adriamycin-treated chemosensitive teratocarcinoma cells. The pattern of inducibility of the gene product correlates well with the p53 status of the cell lines, indicating that expression of the gene is regulated by p53. The cloning and analysis of that cDNA is described in detail below.

From the deduced amino acid sequence of the cDNA, it has been determined that the gene encodes a protein that is a new member of the tumor necrosis factor receptor (TNFR) protein family. This protein is referred to herein as "Killer" or "Killer protein", in view of its presumed role in induction of apoptosis. The genomic DNA or cDNA that encodes the Killer protein is sometimes referred to herein as the "*killer*" gene or cDNA.

The TNFR family includes two TNFRs (TNFR1 and TNFR2), the receptor for lymphotoxin-β, nerve growth factor (NGF) receptor (p75), CD40, CD27, CD30, DR3 (death receptor-3), DR4, HVEM (herpes virus early mediator), and

CAR1 (cytopathic avian leukosis-sarcoma virus receptor. See Beutler, B. & Van Huffel, *Ann. NY Acad. Sci.*, 730:118-133 (1994), S. Nagata, *Cell*, 88:355-365 (1997), G. Pan, et al., *Science*, 276:111-113 (1997). The Killer protein of the invention shares general structural features of the TNFR family in that it is believed to comprise three domains: (1) an extracellular domain at the amino terminus; (2) a transmembrane domain in the central region; and (3) a carboxyl "death" domain. Functionally, Killer is believed to behave in general like a TNFR: the extracellular domain functions as receptor for a cognate extracellular ligand (analogous to TNF for TNFR1); binding of the ligand to the extracellular receptor domain initiates a conformational change through the transmembrane domain to the death domain, which transduces a signal (or cascade of signals) within the cell that ultimately induces apoptosis.

A comparison of Killer with the closely related members of the TNFR (specifically focusing on the death domain) family is set forth in greater detail in Example 1 and shown in Figures 1B and 2. As can be seen, though Killer shares sufficient amino acid sequence similarity with other members of the TNFR family to warrant including it within that family, it is a unique member of that family. The closest homolog is DR4, which shares only about 64% amino acid sequence identity with Killer, even in the most conserved death domain, and about 60% identity comparing all of SEQ ID NO:2, which comprises the death domain, the transmembrane domain and the extracellular domain. Moreover, expression of the *Killer* gene appears to be regulated by p53, presumably through one or more p53 binding sites. The gene that encodes DR4 has not been reported to be regulated by p53.

Killer also appears to be unique in that it is regulated by DNA damage. Results obtained by the present inventor demonstrate that, whereas Killer mRNA expression is induced by DNA damage in wild-type p53 containing

cells, so such universal phenomenon was observed for either DR4 or the decoy receptor, TRID.

Loss of Killer's apoptotic function has also been linked to a human cancer. Transfections of head and neck squamous cell carcinoma, colon and ovarian carcinoma cell lines revealed loss of growth-suppressive function associated with a *Killer* truncation mutant derived from head and neck tumor.

Example 2 describes further important characteristics of Killer. In that Example it is shown that, unlike the *p53*-target CDK-inhibitor *p21*$^{WAF1/CIP1}$, Killer is only induced in cells undergoing *p53*-dependent apoptosis and not cell cycle arrest. Inhibition of transcription by Actinomycin D blocks both *Killer* and *p21* gene induction in cells undergoing *p53*-dependent apoptosis, indicating that *p53* regulation of *Killer* is transcriptional.

The DNA of the invention encodes a Killer protein. The gene, its corresponding cDNA and encoded protein have been designated Killer due to its involvement in the apoptotic pathway. The gene is turned on early in the dying process in chemotherapy treated chemosensitive, but not chemoresistant ovarian cancer cells. Apoptosis is a type of cell death that is thought to be under direct genetic control. During apoptosis, cells lose their cell junctions and microvilli. The cytoplasm condenses and nuclear chromatin marginates into a number of discrete masses. While the nucleus fragments, the cytoplasm contracts and mitochondria and ribosomes become densely compacted. After dilation of the endoplasmic reticulum and its fusion with the plasma membrane, the cell breaks up into several membrane bound vesicles, also known as apoptotic bodies, which are usually phagocytosed by adjacent cells.

The death domain of Killer is highly homologous to such domains in other known death receptors that cause apoptosis in response to a variety of other signals. In

accordance with the present invention, it has also been shown that the expression of the death domain in *E. coli* is toxic.

Figure 1 sets forth the nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of a cDNA of human origin encoding Killer protein of the invention. SEQ ID NO:1 comprises the complete coding sequence of Killer. The protein contains a transmembrane domain, a C-terminal death domain, and an N-terminal extracellular domain. This sequence information indicates that Killer may be classified as a new member of the TNFR family, as discussed above.

Although the human Killer-encoding gene is described and exemplified herein, the present invention also encompasses nucleic acid sequences and proteins from other species that are functionally and structurally homologous to be used interchangeably with Killer-encoding nucleic acids and proteins for the diagnostic, therapeutic, and research purposes described below. Because of the high degree of conservation of genes encoding specific signal transducers and related oncogenes, it will be appreciated by those skilled in the art that, even if the interspecies Killer similarity is low, Killer-encoding nucleic acids and Killer proteins from a variety of mammalian species should possess a sufficient degree of homology with Killer so as to be interchangeably useful with Killer in such diagnostic and therapeutic applications. Accordingly, the present invention is drawn to mammalian Killer-encoding nucleic acids and Killer proteins, preferably to Killer of primate origin, and most preferably to Killer of human origin. Accordingly, when terms such as "Killer" or "Killer-encoding nucleic acid" are used herein, they are intended to encompass mammalian Killer-encoding nucleic acids and Killer proteins falling within the confines of homology set forth below, of which human Killer is an exemplary member.

Allelic variants and natural mutants of SEQ ID NO:1 are likely to exist within the human genome and within the genomes of other mammalian species. Because such variants are expected to possess certain differences in nucleotide and amino acid sequence, this invention provides an isolated nucleic acid molecule and an isolated Killer protein having at least about 50-60% (preferably 60-80%, most preferably over 80%) sequence homology in the coding region with the nucleotide sequence set forth as SEQ ID NO:1, and the amino acid sequence of SEQ ID NO:2. Such nucleic acid sequences will also specifically hybridize with SEQ ID NO:1 or its complement.

Because of the natural sequence variation likely to exist among Killer proteins and nucleic acids encoding them, one skilled in the art would expect to find up to about 40-50% sequence variation, while still maintaining the unique properties of the Killer protein of the present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the protein. Accordingly, such variants are considered substantially the same as one another and are included within the scope of the present invention.

For purposes of this invention, the term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variations that are conservative or neutral. With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative

substitutions and/or variations in regions of the polypeptide. The terms "percent identity" and "percent similarity" are also used herein in comparisons among amino acid sequences. These terms are intended to be defined as they are in the UWGCG sequence analysis program (Devereaux et al., Nucl. Acids Res. 12: 387-397, 1984), available from the University of Wisconsin.

The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") are used.

I. Preparation of Killer-Encoding Nucleic Acid Molecules, Killer Proteins and Antibodies Immunologically Specific for those Proteins

A. Nucleic Acid Molecules

Nucleic acid molecules encoding the Killer proteins of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence information, such as the cDNA having SEQ ID NO:1, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA

molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a 4.44 kb double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

Nucleic acid sequences encoding Killer may be isolated from appropriate biological sources using methods known in the art. In a preferred embodiment, a cDNA clone is isolated by subtractive hybridization of a cDNA library of human origin. In an alternative embodiment, human genomic clones encoding Killer may be isolated. In fact, genomic clones of Killer have been isolated (an exemplary clone, P1-Killer, is shown in Fig. 9), and a complete physical map of the *Killer* gene, located on human chromosome 8p21, has been constructed (Figure 8). Alternatively, *Killer* cDNA or genomic clones from other mammalian species may be obtained.

In accordance with the present invention, nucleic acids having the appropriate level sequence homology with a selected region of SEQ ID NO:1 (e.g. the segment encoding the transmembrane domain or the death domain) may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six

hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6\text{Log}[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$

As an illustration of the above formula, using [N+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such sequences would be considered substantially complementary to the probe.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a suitable *E. coli* host cell. Optionally, the vectors may contain inducible promoter sequences, such as metallothionine, tetracycline or dexamethasone responsive promoters. Such vectors enable the investigator to regulate the expression of killer protein in the transformed tumor cell while assessing induction of apoptosis.

Killer-encoding nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or

antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the cDNA having SEQ ID NO:1. Such oligonucleotides are useful as probes for detecting *Killer* genes in test samples of potentially malignant cells or tissues, e.g. by PCR amplification, or for the isolation of homologous regulators of apoptosis.

In addition, 5′ and 3′ regulatory sequences of *Killer*, available on the genomic clone discussed above, can be used to advantage. For instance, the promoter sequence can be cloned upstream of a reporter gene, so that drug screening can be performed to isolate compounds that induce *Killer's* expression. Reporters may include LacZ, Luciferase, GFP, Hygromycin, or other negative or positive selection markers.

B. Proteins

A Killer protein of the present invention may be prepared in a variety of ways, according to known methods. The protein may be purified from appropriate sources, e.g., human or animal cultured cells or tissues, by immunoaffinity purification. However, this is not a preferred method due to the low amount of protein likely to be present in a given cell type at any time.

The availability of nucleic acids molecules encoding Killer enables production of the protein using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

Alternatively, according to a preferred

embodiment, larger quantities of Killer may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule, such as the cDNA having SEQ ID NO:1 may be inserted into a plasmid vector adapted for expression in a bacterial cell, such as *E. coli*, or into a baculovirus vector for expression in an insect cell. Such vectors provide the regulatory elements necessary for expression of the DNA in the given host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

The Killer protein produced by gene expression in a recombinant procaryotic or eucyarotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

The Killer proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, such proteins may be subjected to amino acid sequence analysis, according to known methods.

The present invention also provides antibodies which bind immunospecifically to Killer proteins may be prepared according to standard methods. In a preferred embodiment, monoclonal antibodies are prepared, which react immunospecifically with various epitopes of Killer. Monoclonal antibodies may be prepared according to

general methods of Köhler and Milstein, following standard protocols. Polyclonal or monoclonal antibodies that immunospecifically interact with Killer can be utilized for identifying and purifying such proteins. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules. Other uses of anti-Killer antibodies are described below.

II. Uses of Killer-Encoding Nucleic Acids, Killer Proteins and Antibodies Thereto

Cellular signaling molecules, including proteins involved in apoptosis pathways, have received a great deal of attention as potential mediators of selective killing of tumor cells for the elimination of cancer from the body. As a signaling molecule involved in p53-mediated apoptosis, Killer and related proteins from other mammalian species will be particularly useful as diagnostic and therapeutic agents. Such molecules will also provide valuable research tools.

A. Killer-Encoding Nucleic Acids

Killer-encoding nucleic acids may be used for a variety of purposes in accordance with the present invention. Killer-encoding DNA, RNA, or fragments thereof may be used as probes to detect the presence of and/or expression of genes encoding Killer, as well as to identify alterations of killer, resulting in loss of function, as they arise in various cancers. In this regard, it should be noted that the diagnostic value of detecting *Killer* expression, even at the mRNA level, is significant. *Killer* DNA may be hypermethylated or simply not activated after exposure to chemotherapy in tumors that go on to become treatment-resistant. Such

information may be useful in designing chemotherapy for particular patients. Methods in which Killer-encoding nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

The Killer-encoding nucleic acids of the invention may also be utilized as probes to identify related genes either from humans or from other species. As is well known in the art, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. Thus, Killer-encoding nucleic acids may be used to advantage to identify and characterize other genes of varying degrees of relation to Killer, thereby enabling further characterization of the signaling cascade involved in the control of apoptosis in different cell types. Additionally, they may be used to identify genes encoding proteins that interact with Killer (e.g., by the "interaction trap" technique), which should further accelerate elucidation of these cellular signaling mechanisms (Golemis, et al., Interaction trap/two-hybrid systems to identify interacting proteins, Unit 20.1.1-20.1.28 in Current Protocols in Molecular Biology, eds. F.M. Ausubel, et al. John Wiley & Sons, NY (1996). Yeast two-hybrid screening may be used to identify the extracellular ligand(s) for Killer, and the proteins that interact with the cytoplasmic portions of Killer, including death domain-containing protein partners. Killer may function to promote apoptosis through a pathway analogous to the Fas and TNFR1 systems. The Fas apoptosis pathway is activated by the binding of Fas ligand (FasL) to Fas, and TNF-induced apoptosis begins by ligation of TNF to TNFR1. The use of interaction trap/two-hybrid assays will permit the identification of Killer-specific, extracellular ligands

which trigger the Killer apoptosis cascade. Further, these assays will be used to identify the intracellular proteins that interact with Killer to potentiate the signal within the cell.

Nucleic acid molecules, or fragments thereof, encoding Killer may also be utilized to control the expression of Killer, thereby regulating the amount of protein available to participate in apoptosis signaling pathways. Alterations in the physiological amount of Killer protein may act synergistically with chemotherapeutic agents used to treat cancer.

In one embodiment, the nucleic acid molecules of the invention may be used to decrease production of Killer protein in a population of cells. In this embodiment, Killer proteins would be made unavailable for the transduction of specific apoptosis signals, providing a model system for research purposes. In this embodiment, antisense oligonucleotides are employed which are targeted to specific regions of Killer-encoding genes that are critical for gene expression. The use of antisense oligonucleotides to decrease expression levels of a pre-determined gene is known in the art. In a preferred embodiment, such antisense oligonucleotides are modified in various ways to increase their stability and membrane permeability, so as to maximize their effective delivery to target cells *in vitro* and *in vivo*. Such modifications include the preparation of phosphorothioate or methylphosphonate derivatives, among many others, according to procedures known in the art. Antisense molecules may also be introduced on conventional DNA vectors used for gene therapy. Once in the appropriate target cells, the antisense molecule is produced by expression of a recombinant DNA segment under control of appropriate promoters and other regulatory sequences.

In another embodiment designed to decrease Killer expression or activity, inhibitors of such expression or activity are identified and utilized. Such

inhibitors would be useful not only for laboratory studies, but also therapeutically, as agents that protect normal tissue against the cytotoxic effects of chemotherapy. A similar approach is being taken, for example, using expression of the MDR1 glycoprotein in bone marrow cells. The MDR1 glycoprotein has been found to be overexpressed in tumor cells that have become resistant to chemotherapeutic agents. Inhibitors of MDR1 are useful to counteract this effect. Similarly, if Killer mutates in any tumors and such mutant Killer interferes with cell death pathwayss, then inhibitors of Killer would be of direct therapeutic value in such cases.

In another embodiment, overexpression of Killer is induced in a target population of cells to generate an excess of Killer molecules. This excess production of Killer may be monitored with immunofluorescence or any other standard technique known in the art. Further, excess production of Killer protein will trigger apoptosis and facilitate the isolation and characterization of the intracellular death domain-containing proteins that interact with Killer and potentiate the apoptosis signal.

Another embodiment of the invention is excess production of the Killer death domain in target cells to assess the effect of the death domain on apoptosis signaling and chemosensitivity.

Another use of the nucleic acids of the invention is in gene replacement therapy for expression of Killer in instances of null or mutant p53 status in a particular target tumor, such that apoptosis may be triggered in tumor cells that will not die in response to chemotherapy. Gene therapy to provide a functional Killer to cells lacking the functional protein is also contemplated. This sort of gene therapy should prove particularly useful in cancers (such as the head and neck cancer described above) specifically associated with a

disrupted *Killer* gene.

As described above, Killer-encoding nucleic acids are also used to advantage to produce large quantities of substantially pure Killer protein, or selected portions thereof. In a preferred embodiment, the C-terminal death domain of Killer (see Figure 1B) is produced by expression of a nucleic acid encoding the domain. The full-length protein or selected domain is thereafter used for various research, diagnostic and therapeutic purposes, as described below.

B. Killer Protein and Antibodies

Purified Killer, or fragments thereof, may be used to produce polyclonal or monoclonal antibodies which also may serve as sensitive detection reagents for the presence and accumulation of Killer (or complexes containing Killer) in cultured cells or tissues from living patients (the term "patients" refers to both humans and animals). Recombinant techniques enable expression of fusion proteins containing part or all of the Killer protein. The full length protein or fragments of the protein may be used to advantage to generate an array of monoclonal antibodies specific for various epitopes of the protein, thereby providing even greater sensitivity for detection of the protein in cells or tissue.

Polyclonal or monoclonal antibodies immunologically specific for Killer may be used in a variety of assays designed to detect and quantitate the protein, which may serve as a prognostic indicator for malignant disease. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization in Killer in cultured cells or biopsy tissue; and (3) immunoblot analysis (e.g., dot blot, Western blot) of extracts from various cells and tissues. Additionally, as described above, anti-Killer antibodies can be used for purification of Killer (e.g.,

affinity column purification, immunoprecipitation).

Anti-Killer antibodies may also be utilized as therapeutic agents to block the normal functionality of Killer in a target cell population. Thus, similar to the antisense oligonucleotides described above, anti-Killer antibodies may be delivered to a target cell population by methods known in the art (i.e. through various lipophilic carriers that enable delivery of the compound of interest to the target cell cytoplasm) where the antibodies may interact with intrinsic Killer protein to render it nonfunctional.

Alternatively, the possibility exists that anti-Killer antibodies may in fact activate Killer's receptor and have a therapeutic value in inducing apoptosis. Precedent exists for such a function, for instance, with anti-Fas antibodies. An anti-Killer antibody use for such a purpose may prove superior to the true ligand, and it may be deliverable intravenously, depending on its specificity for cancer cells, and absence of toxicity. The antibody also may be injected directly into a tumor.

From the foregoing discussion, it can be seen that Killer-encoding nucleic acids and antibodies to Killer proteins of the invention can be used to detect gene expression and protein accumulation for purposes of assessing the genetic and protein interactions involved in the regulation of apoptosis in both wild-type p53 positive and negative cells.

Aberrant signal transduction in cells is often correlated with cellular transformation and cancer of various tissue types. It is expected that these tools will be particularly useful for diagnosis and prognosis of human neoplastic disease as described above. Potentially of greater significance, however, is the utility of Killer-encoding nucleic acids, proteins and antibodies as agents to assess and alter the chemosensitivity of a targeted tumor cell. In any of

their therapeutic uses, the nucleic acids, proteins and antibodies of the invention can be used alone, or as adjuncts to other therapeutic agents.

Although the compositions of the invention have been described with respect to human diagnostics and therapeutics, it will be apparent to one skilled in the art that these tools will also be useful in animal and cultured cell experimentation with respect to various malignancies and/or other conditions manifested by altered patterns of apoptosis or lack of apoptosis in response to DNA damaging agents or ionizing radiation. They can also be used to generate animal model systems, e.g. "knockout" animals that do not express the gene, as a model of cancer susceptibility. Such animals are useful as models for human disease and treatment thereof, inasmuch as they may exhibit phenotypes that may mimic a developmental abnormality and may have a predisposition to cancer, especially following exposure to carcinogens. In connection with this embodiment, it is preferable to clone a mouse homolog of *Killer*, according to standard methods as described above.

The following Examples are provided to describe the invention in further detail. The examples are intended to illustrate and not to limit the invention.

EXAMPLE 1
Isolation and Characterization of a Nucleic Acid Molecule Encoding Human Killer Protein

In this Example the cloning of a cDNA molecule encoding human Killer is described. To identify genes transcriptionally regulated by wild-type p53 in response to DNA damage, a cDNA library enriched for the presence of such genes was constructed and screened by a subtractive hybridization technique. The cell line used as a source for the cDNA library was PA-1, a human, wild-type p53-positive, ovarian teratocarcinoma. p53 status

correlates with chemosensitivity in these cells (Wu & El-Deiry, Nature Med. 2: 255-256, 1996).

Materials and Methods

Cell lines and culture conditions. The human leukemia cell line ML-1, a gift from Michael B. Kastan (Johns Hopkins University, Baltimore, MD), the human lung cancer cell line H460, a gift from Stephen B. Baylin (Johns Hopkins University, Baltimore, MD) and the human colon carcinoma cell line HCT-116, a gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (Wu et al., Clin. Cancer Res. 2: 623-633, 1996; Prabhu et al., Clin. Cancer Res. 2: 1221-1229, 1996). The human ovarian cancer cell lines PA-1 and SKOV3, and the human breast cancer line SKBr3 were obtained from the American Type Culture Collection (Rockville, MD) and maintained as previously described (Wu et al., 1996, *Supra;* Blagosklonny & El Deiry, Int. J. Cancer 67: 386-397, 1996). The human colon carcinoma cell line SW480 was obtained from the University of Pennsylvania Cell Center and maintained as previously described (Fuchs et al., Cancer Res. 57: 2550-2554, 1997). HPV-E6 expressing clones of HCT-116, PA-1, and H460 were transfected and maintained as previously described (Wu & El Deiry, Nature Med. 2: 255-256, 1996; Wu et al., 1996, *supra;* Prabhu et al., 1996, *supra*). Cells were grown in the presence of 0.3 μg/ml adriamycin for 10 hrs followed by isolation of total RNA as previously described (El Deiry et al., Cell 75: 817-825, 1993). H460-neo and H460-E6 cells were irradiated at 500 rads and grown for 10 hrs followed by isolation of total RNA as previously described (Fuchs et al., 1997, *supra;* El Deiry et al., 1993, *supra*). Replication-deficient adenovirus recombinants expressing β-galactosidase (Ad-LacZ) or wild-type *p53* (Ad-p53) were gifts from Dr. B. Vogelstein (Johns Hopkins University),

and were prepared, titered, and used as previously described (Fuchs et al., 1997, *supra*; Blagosklonny & El Deiry, 1996, *supra*; El Deiry et al., 1993, *supra*). Cell lines were infected at a multiplicity of infection (MOI) of 50, and total protein and RNA were harvested 20 hrs later. The infectivity of the cell lines was determined by X-gal staining, as previously described (Blagosklonny & El Deiry, 1996, *supra*).

Subtractive hybridization screening. PA1-neo ovarian teratocarcinoma cells (Wu & El Deiry, 1996, *supra*) were incubated for 10 hrs in the presence of 0.3 μg/ml adriamycin. A cDNA library was constructed using poly(A+) RNA prepared from the adriamycin-treated cells. A total of 50,000 plaques were differentially screened by hybridization to the following: (1) a cDNA probe prepared from equal portions of RNA isolated from untreated PA1-neo cells and PA1-E6 cells, and (2) a subtracted probe prepared by using 500 ng cDNA from adriamycin-treated PA1-neo cells as target and a total of 20 μg driver poly (A+) RNA (10 μg from untreated PA1-neo cells and 10 μg from adriamycin-treated PA1-E6 cells). The cDNA library and probes were prepared using previously described methods (El Deiry et al., 1993).

The initial isolate of the *Killer* cDNA was 0.6 kb of the extreme 3'-untranslated region and this hybridized to a 4.4 kb mRNA species. Hybridization of this probe to the original oligo dT-primed adriamycin-treated PA1 cDNA library yielded a 2.0 kb fragment containing 3'-untranslated *Killer* sequence with no homology in the Genbank database. We performed 5'-RACE PCR to obtain the 5'-coding sequence of *Killer* using the Marathon™ cDNA Amplification Kit (Clontech).

N rthern Blot Analyses. Northern blot analyses were performed using either 10 μg of total cellular RNA (Figs. 1A,3) or 2 μg poly (A+) RNA (Multiple Tissue Northern (MTN™) Blot from Clontech) as previously described. The PA1-neo/E6 northern blot was probed with

a 600 bp 3′ untranslated region of the *Killer* cDNA. All other northern blots were probed with a 1.14 kb fragment from the C-terminal coding region of the *Killer* cDNA. In addition, a 2.0 kb human β-actin probe (Clontech) was used to probe the MTN™ blot. p21 mRNA expression was detected as previously described (Fuchs et al., 1996, *supra*; El Deiry et al., 1993, *supra*).

Plasmids. The full-length open reading frame of the *Killer* cDNA was amplified by RT-PCR from adriamycin-treated PA1 cellular RNA using 5′-CCCAAGCTTCCGCCATGGAACAACGGGGAC-3′ (SEQ ID NO:8) and 5′-CGCGGATCCTTAGGACATGGCAGAGTCTGC-3′ (SEQ ID NO:9) and the following PCR conditions: 35 cycles of denaturation at 95°C for 30 seconds followed by annealing at 68°C for 90 seconds and extension at 72°C for 60 seconds. This PCR product was subcloned into the pCRII vector using the TA-cloning kit (Invitrogen) and followingrestriction by BamH1 and HindIII the cDNA was cloned into the mammalian expression vector pCEP4 (Invitrogen). The cytoplasmic domain was obtained by PCR amplification (30 cycles: 95°C for 30 sec, 65°C for 1 min, 72°C for 1 minute) using *Killer* cDNA as template and the following primers: 5′-CGCGGATCCGTCCTCAATCTTCTGCTTGG-3′ (SEQ ID NO:10)and 5′-CCCAAGCTTCCACCATGGTTGCAGCCGTAGTCTTG-3′. (SEQ ID NO:11). The death domain was amplified using the primers 5′-CCCAAGCTTCCACCATGGACTCCTGGGAGCCGC-3′ (SEQ ID NO:12) and 5′-CGCGGATCCGTCCTCAATCTTCTGCTTGG-3′ (SEQ ID NO:13) and the same PCR conditions as the cytoplasmic domain. The PCR products were restricted with BamH1 and HindIII and subcloned into pCEP4. For *in-situ* RNA hybridization, nucleotides 106 to 726 of *Killer* (Figure 1) by PCR amplification and TA-cloning into the pCR™II (Invitrogen) vector in either sense or antisense orientation.

Transf ctions and apoptosis assays. Transfection of SW480 cells was carried out as previously described (El Deiry et al., 1993, *supra*). TUNEL assays

were performed using SW480 cells transfected in 8-well chamber slides. After 36 hrs, TUNEL-positive cells were detected using the Apoptag Plus *In-situ* apoptosis detection kit-Fluorescein (ONCOR). Transfection efficiency in these experiments was 20%.

Chromosomal localization. Fluorescence *in-situ* hybridization (FISH) was performed by standard techniques as previously described (Krantz et al., Am. J. Med. Genet. 70: 80-86, 1997). Three P1 clones (Genome Systems) containing the *Killer* gene were labeled by nick translation with biotin-11-dUTP and hybridized to metaphase spreads from a normal individual. Chromosomes were counter-stained with 4,6-diamino-2-phenyl-indole (DAPI). Analysis was carried out on a Vysis imaging system and after FISH, DAPI staining was computer converted to a G-banding image for chromosome and band identification.

In-situ mRNA expression. *In-situ* hybridization of frozen sections was performed using a modified version of the digoxigenin labeled method as described (Panoskaltsis-Mortari & Bucy, BioTech 18: 300-307, 1995). In brief, colonic tissue was snap frozen in OCT and stored at -80°C. Five μm sections were cut and placed on Probe-On slides (Fisher Scientific, Pittsburgh, PA). The tissue sections were thawed at room temperature for 1-2 minutes and fixed in 3% paraformaldehyde for 1 minute at room temperature. After one wash with 2 x SSC, the slides were incubated for 8 minutes in 0.2 M HCl and rinsed with 0.1 M triethanolamine, pH 8.0. The slides were then incubated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 15 minutes followed by a rinse with 2 x SSC. Pre-hybridization was performed at 50°C for 30 minutes in a solution containing 50% formamide, 4 x SSC, 1 x Denhardt's, 10% dextran sulfate, 500 μg/ml of heat denatured salmon sperm DNA, and 250 μg/ml of yeast tRNA. The slides were then hybridized in pre-hybridization solution containing heat-denatured

(80°C) RNA probe at 50°C overnight. Following hybridization, the slides were rinsed in 2 x SSC for 5 minutes and STE (500 mM NaCl, 20 mM tris-HCl, pH 7.5, 1 mM EDTA) for 1 minute. After incubation in RNase A (40 μg/ml in STE) for 30 minutes at 37°C, the slides were washed with 2 x SSC containing 50% formamide for 5 minutes at 50°C followed by three washes with 1 x SSC and 0.5 x SSC, each for 5 minutes. Immunological detection of digoxigenin was carried out using a sheep-anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN). Digoxigenin labeled RNA probes for *Killer* (both sense and anti-sense) were transcribed from the T7 promoter of pCR™II (Invitrogen), after linearization by digestion with HindIII, using the Riboprobe Gemini System 2 kit (Promega) and the Dig/Genius™4 RNA Labeling Kit (SP6/T7; Boehringer Mannheim).

Targeting p53 for destruction. To generate a tumor cell line in which p53 function was lost, PA-1 cells were transfected with a human papilloma virus (HPV) 16 E6 expression plasmid that expresses E6 protein constitutively driven by the cytomegalovirus (CMV) immediate early promoter. PA-1 cells were transfected with either pCMV-neo-bam (referred to herein as either PA-1/neo or "wild-type") or pCMV16E6 (referred to herein as either PA-1/E6 or "degraded" to reflect the function of the E6 protein in degrading p53), as described previously (El-Deiry et al., 1993, *supra*). Individual clones were isolated following selection in the presence of 500 μg/ml G418 (Life Technologies, Inc.), as described by Baker et al., Science 249: 912-915 (1990).

Expression of the Death Domain of Killer in *E. coli*. *E. coli* cells were transformed with a pGEX vector expressing the death domain of the killer protein. Bacterial cells were seeded at low density and grown up overnight in L broth + ampicillin. SDS-PAGE was performed which confirmed that the protein was expressed

following induction with IPTG. The toxicity assay was performed in the presence and absence of glucose to rule out catabolite repression effects. Toxicity of the death domain was assessed by measuring absorbance at 520 nM in a spectrophotometer over a 5 hour time course.

Results

The *p53* tumor suppressor is the most commonly mutated gene in human cancer, and inherited mutations lead to the cancer-prone Li-Fraumeni syndrome. There is evidence that *p53* is a key determinant of the sensitivity of mammalian cells to the cytotoxic effects of chemo- and radio-therapy. Biochemical studies have demonstrated that the majority of tumor-derived mutants of *p53* have lost the ability to activate gene expression, suggesting that transcriptional activation by p53 may be critical for its role in the control of cell proliferation. Stabilization of p53 protein following virus infection, nucleotide depletion, hypoxia or DNA damage leads to cell cycle arrest and/or apoptosis. Cell cycle arrest is believed to be mediated by transcriptional activation of $p21^{WAF1/CIP1}$, a universal inhibitor of cyclin-dependent kinases and PCNA-dependent processive DNA replication. p53 upregulates expression of several genes involved in apoptosis, including *bax* and *Fas/APO1*. However, the fact that neither *bax*- nor *Fas/APO1*-null cells are deficient in DNA damage-induced (*p53*-dependent) apoptosis suggests that other mechanisms likely contribute to this process.

We previously found that targeted degradation of the p53 protein by HPV16 *E6* overexpression in PA1 ovarian teratocarcinoma cells led to inhibition of adriamycin- induced apoptosis and chemoresistance. In order to explore the pathway of cell death in the chemosensitive PA1 cells, we carried out subtractive hybridization screening of a library enriched for adriamycin-induced transcripts (see Methods). Of the

50,000 phage clones representing PA1 mRNA's expressed at 10 hrs following adriamycin treatment, 100 appeared to hybridize more strongly to a subtracted vs. control probe in primary screening. In secondary screening, 30 of the initially chosen 100 plaques reproducibly displayed differential subtractive hybridization and were then used individually as probes of Northern blots carrying RNA from untreated vs. adriamycin-treated PA1 cells. *p21*$^{WAF1/CIP1}$ was one of six clones whose expression was increased following adriamycin treatment, and its presence provided an indication that the subtraction had yielded at least one expected target. Another known gene that was isolated was *Cathepsin D*, a gene encoding a protease that was also recently isolated as an antisense suppressor of cytokine-induced apoptosis. Of the other four (novel) cDNA's, one appeared to be strongly induced by adriamycin (doxorubicin) and, thus, was further characterized. Database analysis revealed that this novel gene (Figure 1) is a member of the TNF-receptor family. Based on the presence of a cytoplasmic death domain (Figure 1, induction of its expression by cytotoxic chemotherapy (Figure 3) and induction of apoptosis by its overexpression, the novel gene was named *Killer*.

The *Killer* gene encodes a 411 amino acid polypeptide (Figure 1) of predicted molecular weight of 45 kD. The protein contains a cytoplasmic C-terminal death domain, a transmembrane domain, and a cysteine-rich extracellular N-terminal domain characteristic of the TNF-receptor family. At the protein level, Killer's closest homolog is DR4 with 55% identity and an additional 9% similarity over a 454 amino acid alignment. See Figure 2A. Figure 2b shows homology between the death domain of *Killer* and other TNF-receptor family members. This alignment revealed that within the death domain, *Killer* shares 64% identity with *DR4*, 28% with *DR3*, 19% with *Fas/APO1*, 29% with *TNFR-1*, and 31% with *CAR1* (Fig. 2B).

We examined the tissue expression pattern of the *Killer* gene by Northern (RNA) blot analysis. Tissues screened were spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. A single transcript of 4.4 kb hybridized to the *Killer* probe and its expression appeared higher in tissues with rapid turnover as compared with nondividing tissues.

Because *Killer* was isolated as an adriamycin-induced transcript, and because adriamycin is a potent inducer of p53 and *p53*-dependent growth suppression, we explored the possibility that *Killer* may be a p53-regulated gene. We examined *Killer* mRNA expression in a series of 11 human cancer cell lines of known p53 status. Figures 3A and 3B show that *Killer* expression was induced following adriamycin exposure only if wild-type *p53* was present but not in cells where it was mutated, degraded, or not expressed. These results strongly correlated *p53*-status with induction of *Killer* gene expression following exposure of (11/11) human cell lines to the DNA damaging Topoisomerase II inhibitor adriamycin. We further found that *Killer* expression could be induced by other DNA damaging agents such as ionizing radiation or etoposide (Fig. 3B for example). In order to more directly test the hypothesis that *Killer* is a *p53*-regulated gene, we infected human cancer cell lines lacking wt *p53* by using a wt *p53*-expressing adenovirus. Fig. 3C shows that in each case tested, *Killer* gene expression was increased by wt *p53* overexpression in a manner similar to *p21* (not shown). These results suggested that *Killer* is a gene whose expression is increased by the *p53* tumor suppressor, even in the absence of DNA damage, raising the possibility that *Killer* may be a mediator in *p53*-dependent apoptosis. This possibility is further supported by the fact that *Killer* expression is elevated early (increased at 10-12

hrs after exposure to adriamycin, gamma-radiation or Ad-p53) in cells that are undergoing apoptosis over a 24-48 hr time course.

The identification of *Killer* as a novel *p53*- and DNA damage-inducible death receptor suggested the possibility that *Killer* inactivation may contribute to tumor development and to resistance to apoptosis *in vivo*. In order to further explore this possibility, we determined the chromosomal localization of the Killer gene by *in-situ* hybridization using as probes three P1-clones containing the gene. Ten metaphases were examined for each P1 clone, and signal was detected on both chromosome 8's in all cells studied. The *in-situ* hybridization indicated that it localizes to chromosome 8p21, a hotspot for translocations in a number of different tumor types. Because recent experiments have demonstrated that the *Type II TGF-β receptor* and the *bax* genes may be mutated in colon cancers with microsatellite instability, we sequenced the death domain of *Killer* from normal DNA and colon cancer cell lines including 8 with microsatellite instability. We found no alteration involving a GAGAGAGA dinucleotide repeat located within the death domain of *Killer* (data not shown).

Because rapidly dividing tissues such as the gut express increased levels of *Killer*, we investigated its *in-situ* mRNA expression to determine if it correlates with apoptosis in human colon. We found that *Killer* is expressed in the proliferating/stem cell compartment of colonic crypt epithelia, a location which correlates with both spontaneous- and irradiation-induced apoptosis. We hypothesized that *Killer* expression may be downregulated in colonic tumor progression as a potential mechanism of inactivation in cancer. We found that *Killer* mRNA expression is low or undetectable in a colonic adenoma and two colon carcinomas with *p53* mutation. In addition, the Northern blot results in Fig. 3A show that the basal expression (in the absence of adriamycin exposure) of

Killer is lower in mutant *p53*-expressing SW480 cells and the p53-degrading HCT116-E6 cells as compared to wild-type *p53*-expressing HCT116-neo cells. It may be that loss of *Killer* expression in colon cancer may be due to *p53* mutation. In this regard, the extreme 5'-end of the *Killer* gene (exon 1) has been previously isolated as a methylated CpG island (Genbank accession # Z66083). DNA methyltransferase expression level has been found to be increased in colonic polyps, at a stage of tumorigenesis that precedes the development of *p53* mutation. It is possible that *Killer* gene expression may be regulated by DNA methylation, an epigenetic phenomenon that has been found to inactivate a number of tumor suppressor genes in cancer.

We investigated the role of *Killer* in apoptosis by transfecting human colon cancer cells with vectors that express the full-length *Killer* cDNA, its cytoplasmic domain, or the death domain. Results showed that overexpression of *Killer*'s death domain is sufficient to induce apoptosis in transfected cells. The apoptosis observed with the *Killer* expression vectors was similar in magnitude to *p53*-induced apoptosis in these cells (35% vs. 32% TUNEL-positive cells).

The death domain of Killer has been cloned into a pGEX vector. Following transformation of *E. coli* cells, and induction of protein expression with IPTG, growth of bacteria was assessed over a 5 hour period. The data in Figure 4 show that expression of the death domain is also toxic to bacteria. This toxicity was not a result of catabolite repression as shown by the +/- glucose controls.

We also investigated the effect of Killer on growth of colon and liver cancer cells. SW480 human colon cancer cells were transfected with either pCEP4 vector or pCEP4-killer, according to protocols described above. Transfected cells were selected with 0.25 mg/ml Hygromycin for two weeks, and stained with Coomassie

Blue. The results of this experiment clearly demonstrated that killer is a potent inhibitor of human colon cancer cell growth: cells transfected with the pCEP4-killer construct were unable to grow, while cells transfected with vector alone were able to grow. Similar results were obtained with HepG2 hepatoblastoma cells.

In summary, we have isolated a novel cell death receptor (*Killer*) whose expression is induced by DNA damaging agents in a p53-dependent manner. *Killer* gene expression is also inducible by wt *p53* overexpression in the absence of DNA damage. Overexpression of *Killer* leads to cell death of cancer cells, and a number of human cancers have decreased expression of the *Killer* gene. Members of the TNF receptor family signal apoptosis through adaptor molecules that directly activate the caspase cascade. The identification of *Killer* provides a potential mechanism by which DNA damaging agents can lead to p53-dependent apoptosis.

EXAMPLE 2
Involvement of Killer in p53-Dependent and -Independent Apoptosis but Not Growth Arrest

Using the subtractive hybridization approach described in Example 1 to isolate adriamycin-inducible transcripts from chemosensitive cells, we cloned the death receptor *Killer* as a DNA damage-inducible p53-regulated gene on chromosome 8p21. Overexpression of Killer induces apoptosis of human cancer cells and eliminates stable colony formation. Because *Killer* is a novel apoptosis-inducing gene that appears to be regulated by p53, we further explore in this Example its regulation in p53-dependent G1-arrest versus apoptosis. Additionally, we examined the mechanism of regulation of *Killer* by p53.

Materials and Methods

Cell Lines and Culture Conditions. The normal human lung fibroblast cell line WI38, the human ovarian

cancer cell line SKOV3, the human breast cancer cell line SKBr3, and the human colon cancer cell line SW480 were obtained from the American Type Culture Collection (Rockville, MD). The human glioblastoma cell line GM (Mercer et al., Proc. Natl. Acad. Sci. USA 87: 6166-6170, 1990) was obtained from Ed Mercer (Thomas Jefferson University).

Adenovirus Infection. The wild-type p53-expressing replication deficient adenovirus Ad-p53 and the LacZ-expressing adenovirus Ad-LacZ (El Deiry et al., 1993, *supra*) were obtained from Bert Vogelstein (Johns Hopkins University). Titering and infection with adenoviruses was carried out at an MOI of 50 as previously described (El Deiry et al., 1993, *supra*; Blagoskonny & El Deiry, 1996, *supra*).

RNA extraction and Northern Blot. RNA extraction and Northern blot were as described in Example 1. The full-length (1.34 kb) human *Killer* cDNA probe was described in Example 1. The full-length human p21WAF1/CIP1, MDM2 and p53 cDNA's are described elsewhere (El Deiry et al., 1993, *supra*). A human bax probe (Oltvai et al., Cell 74: 609-616, 1993; Miyashita et al., Oncogene 9: 1799-1805, 1994; Miyashita et al., Cell 80: 293-299, 1995) was provided by John Reed (Burnham Institute).

Apoptosis assays. GM cells were infected by Ad-LacZ or Ad-p53. After 30 hrs, cells were stained with DAPI as previously described (Blagoskonny & El Deiry, 1996, *supra*).

Determination of messenger RNA stability. SW480 human colon adenocarcinoma cells were infected by either Ad-LacZ or Ad-p53 for one hour using an MOI of 50 as previously described (Blagosklonny & El Deiry, 1996, *supra*; Meng et al, Clin. Cancer Res. 4: 251-259, 1998). After an additional incubation at 37°C for 14 hrs., Actinomycin D was added to the cultures at a final concentration of 30 ng/ml. Total RNA was harvested at

different time points after Actinomycin D addition as indicated in Fig. 4, and Northern blotting was performed as described above.

Results

***Killer* expression is not increased in p53-dependent growth arrest.** One of the major questions in elucidating p53 function has been to understand what determines whether cells undergo cell cycle arrest or apoptosis following p53 activation. We have found a perfect correlation between induction of *Killer* expression and exposure of wild-type p53-expressing human cancer cells to apoptosis-inducing cytotoxic agents (Example 1). Following exposure to lethal doses of agents such as adriamycin, etoposide, or ionizing radiation, only cells which contain wild-type p53 induced *Killer* expression. Cells with mutant or degraded p53 failed to upregulate *Killer* expression in response to genotoxic drugs. In order to investigate whether the p53-dependent upregulation of *Killer* is specific to cells undergoing apoptosis, we used two well established systems where p53 induction leads only to cell cycle arrest.

The human glioblastoma cell line GM contains an endogenous mutant p53 and an exogenous dexamethasone-inducible wild-type p53. Upon addition of dexamethasone to the media, it has been shown that these cells induce expression of wild-type p53 which in turn transcriptionally activates expression of p21, and the cells then undergo cell cycle arrest in G1-phase and not apoptosis. Fig. 5A shows that upon addition of dexamethasone, the expected induction in p21 expression is observed, but there is no accompanying upregulation of *Killer* expression. These results suggest that cells undergoing p53-dependent cell cycle arrest induce p21 as expected but do not induce expression of *Killer*, a putative mediator of p53-dependent apoptosis.

In order to validate these observations in a different system, we chose to examine the expression levels of *p21* and *Killer* in WI38 normal human lung fibroblast cells either with no treatment or following exposure to ionizing radiation. It has been shown previously that the wild-type p53 containing WI38 lung fibroblasts respond to g- irradiation by undergoing a prolonged cell cycle arrest associated with p21 upregulation, and not apoptosis. Following irradiation, p21 expression was induced as expected but *Killer* expression was not increased (Fig. 5B). In fact at 10 Gy, *Killer* mRNA expression appeared to be inhibited. These results are consistent with the hypothesis that the p53-dependent induction of *Killer* is "apoptosis-specific" and not a general response in cells undergoing only p53-dependent cell cycle arrest.

Increased expression of *Killer* in cells undergoing p53-dependent apoptosis. To further investigate the hypothesis that p53-dependent upregulation of *Killer* expression may be a determinant of apoptosis induction, we infected GM cells with Ad-p53. GM cells were found to undergo apoptosis following Ad-p53 as compared to Ad-LacZ infection or dexamethasone treatment (Fig. 5A). Unlike the dexamethasone-treated GM cells which do not undergo p53-dependent apoptosis, the Ad-p53 infected cells upregulated expression of the *Killer* gene. We suspect that the higher levels of p53 achieved following Ad-p53 infection probably contribute to the apoptosis phenotype in GM cells. These results further correlate the p53-dependent induction of *Killer* with apoptosis.

We further investigated the kinetics of Killer upregulated expression following Ad-p53 as compared to Ad-LacZ infection of the SKOV3 human ovarian carcinoma cell line (Fig. 6B). Expression of *Killer* was detectably increased as early as 6 hours following Ad-p53 infection of the SKOV3 cells. This pattern of expression would be

expected for genes involved in p53 mediated apoptosis which occurs over 1-3 days following Ad-p53 infection (Fig. 6A and data not shown).

Transcriptional and post-transcriptional control of TRAIL death receptor expression in p53-dependent apoptosis. We further explored the mechanism by which Killer is induced following p53 overexpression in cancer cells undergoing apoptosis. We infected SKOV3 cells (Fig. 7A) or GM cells (Fig. 7B) using either Ad-LacZ or Ad-p53 for one hour followed by a 20 hr incubation in the presence of the transcription inhibitor Actinomycin D. Expression of the p53 targets *p21*, *MDM2* and *Killer* was greatly increased in Ad-p53 as compared with Ad-LacZ infected SKOV3 cells in the absence of Actinomycin D (Fig. 7A, compare lanes 3 to lanes 1). In the presence of Actinomycin D, the p53-dependent upregulation of *p21*, *MDM2*, and *Killer* was abrogated (Fig. 7A, compare lanes 4 to lanes 2). These results suggest that *Killer*, *p21* and *MDM2* appear to be regulated by p53 at the level of transcription. p53 mRNA levels were also not decreased by Actinomycin D in Ad-p53 infected cells (Fig 7A).

We investigated the effect of Actinomycin D on the expression of *Killer* following infection of GM cells by Ad-p53 or Ad-LacZ (Fig. 3B). The p53- dependent upregulation of *Killer* expression (Fig. 7B lane 3 vs. 1, upper panel) was blocked by Actinomycin D in GM cells (lane 4 vs. 3).

The present invention is not limited to the embodiments specifically described above, but is capable of variation and modification without departure from the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of the University of Pennsylvania

(ii) TITLE OF THE INVENTION: NUCLEIC ACID ENCODING A NOVEL CHEMOTHERAPY-INDUCED PROTEIN, AND METHODS OF USE

(iii) NUMBER OF SEQUENCES: 13

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(C) CITY: Philadelphia
(D) STATE: Pennsylvania
(E) COUNTRY: USA
(F) ZIP: 19103

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Not yet assigned
(B) FILING DATE: 10-JUL-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/052,305
(B) FILING DATE: 11-JUL-1997

(A) APPLICATION NUMBER: US 60/054,710
(B) FILING DATE: 04-AUG-1997

(A) APPLICATION NUMBER: US 60/060,473
(B) FILING DATE: 30-SEP-1997

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(C) REFERENCE/DOCKET NUMBER: PENN J1638PCT

(ix) TELECOMMUNICATION INFORMATION:
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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1139 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GTTGTCGCCG CGGTCCTGCT GTTGGTCTCA GCTGAGTCTG CTCTGATCAC CCAACAAGAC    180
CTAGCTCCCC AGCAGAGAGC GGCCCCACAA CAAAAGAGGT CCAGCCCCTC AGAGGGATTG    240
TGTCCACCTG GACACCATAT CTCAGAAGAC GGTAGAGATT GCATCTCCTG CAAATATGGA    300
CAGGACTATA GCACTCACTG GAATGACCTC CTTTTCTGCT TGCGCTGCAC CAGGTGTGAT    360
TCAGGTGAAG TGGAGCTAAG TCCCTGCACC ACGACCAGAA ACACAGTGTG TCAGTGCGAG    420
AAGGCACCTT CCGGGAAGAA GATTCTCCTG AGATGTGCCG GAAGTGCCGC ACAGGGTGTC    480
CCAGAGGGAT GGTCAAGGTC GGTGATTGTA CACCCTGGAG TGACATCGAA TGTGTCCACA    540
AAGAATCAGG CATCATCATA GGAGTCACAG TTGCAGCCGT AGTCTTGATT GTGGCTGTGT    600
TTGTTTGCAA GTCTTTACTG TGGAAGAAAG TCCTTCCTTA CCTGAAAGGC ATCTGCTCAG    660
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ATGTCCTCAA TGAGATCGTG AGTATCTTGC AGCCCACCCA GGTCCCTGAG CAGGAAATGG    780
AAGTCCAGGA GCCAGCAGAG CCAACAGGTG TCAACATGTT GTCCCCCGGG GAGTCAGAGC    840
ATCTGCTGGA ACCGGCAGAA GCTGAAAGGT CTCAGAGGAG GAGGCTGCTG GTTCCAGCAA    900
ATGAAGGTGA TCCCACTGAG ACTCTGAGAC AGTGCTTCGA TGACTTTGCA GACTTGGTGC    960
CCTTTGACTC CTGGGAGCCG CTCATGAGGA AGTTGGGCCT CATGGACAAT GAGATAAAGG   1020
TGGCTAAAGC TGAGGCAGCG GGCCACAGGG ACACCTTGTA CACGATGCTG ATAAAGTGGG   1080
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro Gly Pro
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        35                  40                  45
Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln
    50                  55                  60
Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
65                  70                  75                  80
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
                85                  90                  95
Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
            100                 105                 110
Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
        115                 120                 125
Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
    130                 135                 140
Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145                 150                 155                 160
Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile
                165                 170                 175
Glu Cys Val His Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala
            180                 185                 190
Ala Val Val Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp
        195                 200                 205
Lys Lys Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly
    210                 215                 220
Asp Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp
225                 230                 235                 240
Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro
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245 250 255
Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val Asn
260 265 270
Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala
275 280 285
Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Glu Gly Asp
290 295 300
Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val
305 310 315 320
Pro Phe Asp Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp
325 330 335
Asn Glu Ile Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr
340 345 350
Leu Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg Asp Ala
355 360 365
Ser Val His Thr Leu Leu Asp Ala Leu Glu Thr Leu Gly Glu Arg Leu
370 375 380
Ala Lys Gln Lys Ile Glu Asp His Leu Leu Ser Ser Gly Lys Phe Met
385 390 395 400
Tyr Leu Glu Gly Asn Ala Asp Ser Ala Met Ser
405 410

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 468 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ala Pro Pro Pro Ala Arg Val His Leu Gly Ala Phe Leu Ala Val
1 5 10 15
Thr Pro Asn Pro Gly Ser Ala Ala Ser Gly Thr Glu Ala Ala Ala Ala
20 25 30
Thr Pro Ser Lys Val Trp Gly Ser Ser Ala Gly Arg Ile Glu Pro Arg
35 40 45
Gly Gly Gly Arg Gly Ala Leu Pro Thr Ser Met Gly Gln His Gly Pro
50 55 60
Ser Ala Arg Ala Arg Ala Gly Arg Ala Pro Gly Pro Arg Pro Ala Arg
65 70 75 80
Glu Ala Ser Pro Arg Leu Arg Val His Lys Thr Phe Lys Phe Val Val
85 90 95
Val Gly Val Leu Leu Gln Val Val Pro Ser Ser Ala Ala Thr Ile Lys
100 105 110
Leu His Asp Gln Ser Ile Gly Thr Gln Gln Trp Glu His Ser Pro Leu
115 120 125
Gly Glu Leu Cys Pro Pro Gly Ser His Arg Ser Glu Arg Pro Gly Ala
130 135 140
Cys Asn Arg Cys Thr Glu Gly Val Gly Tyr Thr Asn Ala Ser Asn Asn
145 150 155 160
Leu Phe Ala Cys Leu Pro Cys Thr Ala Cys Lys Ser Asp Glu Glu Glu
165 170 175
Arg Ser Pro Cys Thr Thr Thr Arg Asn Thr Ala Cys Gln Cys Lys Pro
180 185 190
Gly Thr Phe Arg Asn Asp Asn Ser Ala Glu Met Cys Arg Lys Cys Ser
195 200 205
Thr Gly Cys Pro Arg Gly Met Val Lys Val Lys Asp Cys Thr Pro Trp
210 215 220
Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly Asn Gly His Asn Ile

225 230 235 240
Trp Val Ile Leu Val Val Thr Leu Val Val Pro Leu Leu Leu Val Ala
245 250 255
Val Leu Ile Val Cys Cys Cys Ile Gly Ser Gly Cys Gly Gly Asp Pro
260 265 270
Lys Cys Met Asp Arg Val Cys Phe Trp Arg Leu Gly Leu Leu Arg Gly
275 280 285
Pro Gly Ala Glu Asp Asn Ala His Asn Glu Ile Leu Ser Asn Ala Asp
290 295 300
Ser Leu Ser Thr Phe Val Ser Glu Gln Gln Met Glu Ser Gln Glu Pro
305 310 315 320
Ala Asp Leu Thr Gly Val Thr Val Gln Ser Pro Gly Glu Ala Gln Cys
325 330 335
Leu Leu Gly Pro Ala Glu Ala Glu Gly Ser Gln Arg Arg Arg Leu Leu
340 345 350
Val Pro Ala Asn Gly Ala Asp Pro Thr Glu Thr Leu Met Leu Phe Phe
355 360 365
Asp Lys Phe Ala Asn Ile Val Pro Phe Asp Ser Trp Asp Gln Leu Met
370 375 380
Arg Gln Leu Asp Leu Thr Lys Asn Glu Ile Asp Val Val Arg Ala Gly
385 390 395 400
Thr Ala Gly Pro Gly Asp Ala Leu Tyr Ala Met Leu Met Lys Trp Val
405 410 415
Asn Lys Thr Gly Arg Asn Ala Ser Ile His Thr Leu Leu Asp Ala Leu
420 425 430
Glu Arg Met Glu Glu Arg His Ala Lys Glu Lys Ile Gln Asp Leu Leu
435 440 445
Val Asp Ser Gly Lys Phe Ile Tyr Leu Glu Asp Gly Thr Gly Ser Ala
450 455 460
Val Ser Leu Glu
465

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Trp Lys Glu Phe Val Arg Thr Leu Gly Leu Arg Glu Ala Glu Ile
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Tyr Ala Ala Leu Glu Arg Met Gly Leu Asp Gly Cys Val Glu Asp Leu
50 55 60
Arg
65

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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            20                  25                  30
Gln Leu Leu Arg Asn Trp His Gln Leu His Gly Lys Lys Glu Ala Tyr
        35                  40                  45
Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala
    50                  55                  60
Glu Lys Ile Gln
65
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE: homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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 1               5                  10                  15
Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr
            20                  25                  30
Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr
        35                  40                  45
Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys
    50                  55                  60
Leu Glu Asp Ile Glu
65
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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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Glu Trp Lys Arg Phe Gly Arg Ala Leu Asp Leu Gln Glu Asn Asp Leu
 1               5                  10                  15
Tyr Leu Ala Glu Gln His Glu Arg Val Ser Cys Glu Pro Arg Tyr Gln
            20                  25                  30
Met Leu Asn Thr Trp Leu Asn Gln Gln Gly Ser Lys Ala Ser Val Asn
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35 40 45
Thr Leu Leu Glu Thr Leu Pro Arg Ile Gly Leu Ser Gly Val Ala Asp
50 55 60
Ile Ile Ala
65

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCCAAGCTTC CGCCATGGAA CAACGGGGAC 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCGGATCCT TAGGACATGG CAGAGTCTGC 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGGATCCG TCCTCAATCT TCTGCTTGG 29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCAAGCTTC CACCATGGTT GCAGCCGTAG TCTTG 35

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCCAAGCTTC CACCATGGAC TCCTGGGAGC CGC 33

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGCGGATCCG TCCTCAATCT TCTGCTTGG 29

What is claimed is:

1. An isolated nucleic acid molecule comprising an open reading frame of part or all of a gene located on human chromosome 8p21, which encodes part or all of a mammalian p53-inducible, apoptosis-mediating protein, which comprises an amino-terminal extracellular receptor domain, a transmembrane domain, and a death domain for protein-protein interactions.

2. The nucleic acid molecule of claim 1, which encodes a human protein.

3. The nucleic acid molecule of claim 2, which is DNA.

4. The DNA molecule of claim 3, which is a cDNA comprising a sequence approximately 1.2 kilobase pairs in length that encodes said p53-inducible, apoptosis-mediating protein.

5. The DNA molecule of claim 3, which is a gene, the exons of which comprise said open reading frame.

6. The nucleic acid molecule of claim 2, which encodes a polypeptide having a sequence substantially the same as SEQ ID NO:2.

7. The nucleic acid molecule of claim 6, which encodes SEQ ID NO:2.

8. The nucleic acid molecule of claim 8, which comprises SEQ ID NO:1.

9. A vector for transformation of a mammalian cell, which comprises the nucleic acid molecule of claim 1.

10. A mammalian cell transformed with the vector of claim 9.

11. An oligonucleotide between 10 and 100 nucleotides in length, that specifically hybridizes with a pre-determined portion of the nucleic acid molecule of claim 1.

12. An isolated nucleic acid molecule having a sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) an allelic variant or natural mutant of SEQ ID NO:1;

c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the same as part or all of a polypeptide encoded by SEQ ID NO:1; and

d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2.

13. A protein produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on human chromosome 8p21, said protein being a p53-induced apoptosis-mediating protein having an amino-terminal extracellular receptor domain, a transmembrane domain, and a death domain for protein-protein interactions.

14. The protein of claim 13, comprising an amino acid sequence substantially the same as SEQ ID NO:2.

15. The protein of claim 13, produced by expression of an open reading frame having a sequence substantially the same as SEQ ID NO:1.

16. Antibodies immunologically specific for the protein of claim 13.

17. A protein produced by expression of a sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) an allelic variant or natural mutant of SEQ ID NO:1;

c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the same as part or all of a polypeptide encoded by SEQ ID NO:1; and

d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2.

18. Antibodies immunologically specific for the protein of claim 17.

19. A non-human genetically engineered mammal, which is unable to produce a functional protein normally produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on human chromosome 8p21, said protein being a p53-induced apoptosis-mediating protein having an amino-terminal extracellular receptor domain, a transmembrane domain, and a death domain for protein-protein interactions.

20. The genetically engineered mammal of claim 19, which is a mouse.

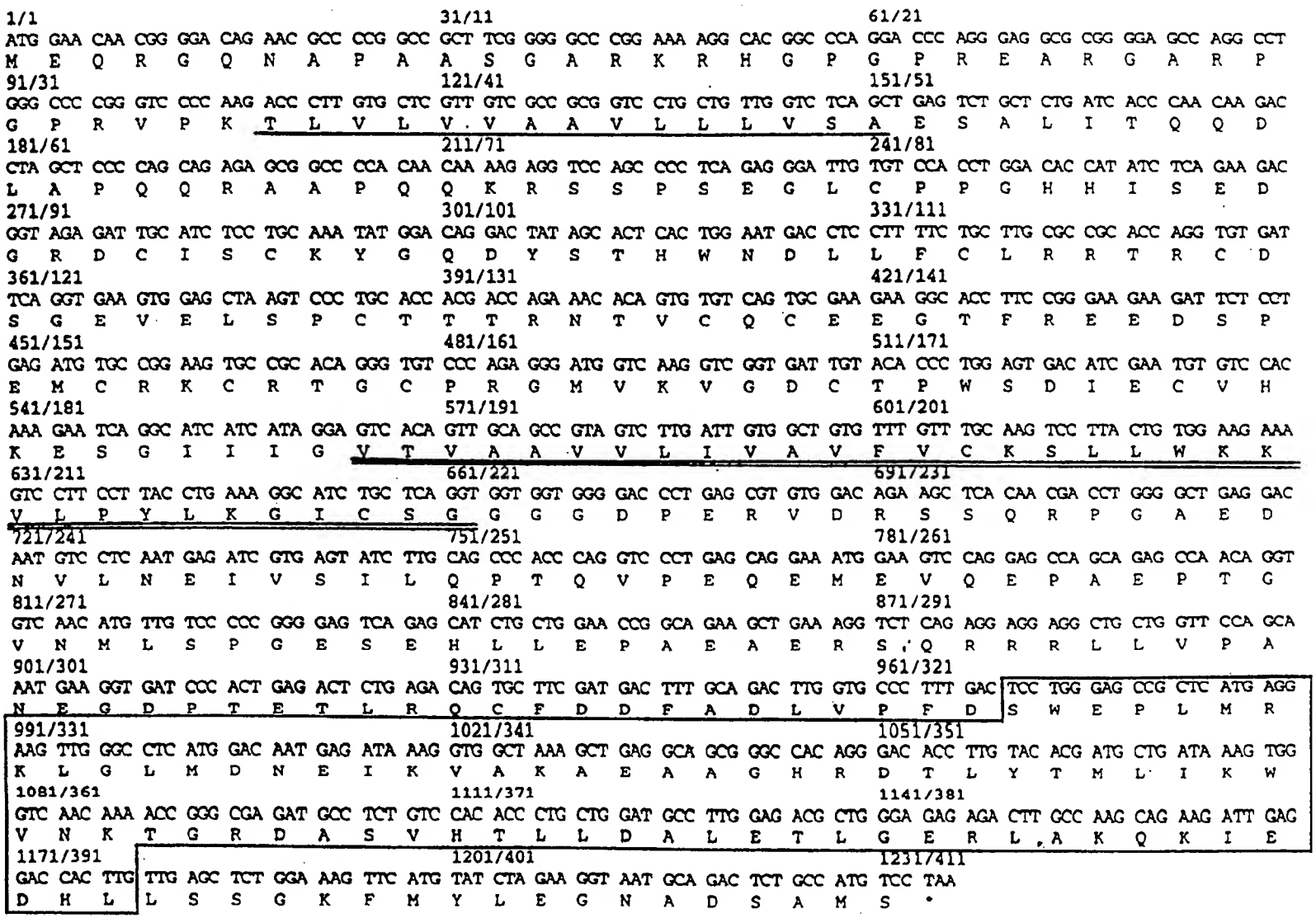

1/1 31/11 61/21
ATG GAA CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG AAA AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC AGG CCT
M E Q R G Q N A P A A S G A R K R H G P G P R E A R G A R P
91/31 121/41 151/51
GGG CCC CGG GTC CCC AAG ACC CTT GTG CTC GTT GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT CTG ATC ACC CAA CAA GAC
G P R V P K T L V L V V A A V L L L V S A E S A L I T Q Q D
181/61 211/71 241/81
CTA GCT CCC CAG CAG AGA GCG GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC
L A P Q Q R A A P Q Q K R S S P S E G L C P P G H H I S E D
271/91 301/101 331/111
GGT AGA GAT TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC TGG AAT GAC CTC CTT TTC TGC TTG CGC CGC ACC AGG TGT GAT
G R D C I S C K Y G Q D Y S T H W N D L L F C L R R T R C D
361/121 391/131 421/141
TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG GAA GAA GAT TCT CCT
S G E V E L S P C T T T R N T V C Q C E E G T F R E E D S P
451/151 481/161 511/171
GAG ATG TGC CGG AAG TGC CGC ACA GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA CCC TGG AGT GAC ATC GAA TGT GTC CAC
E M C R K C R T G C P R G M V K V G D C T P W S D I E C V H
541/181 571/191 601/201
AAA GAA TCA GGC ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT GTG GCT GTG TTT GTT TGC AAG TCC TTA CTG TGG AAG AAA
K E S G I I I G V T V A A V V L I V A V F V C K S L L W K K
631/211 661/221 691/231
GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT GGG GCT GAG GAC
V L P Y L K G I C S G G G G D P E R V D R S S Q R P G A E D
721/241 751/251 781/261
AAT GTC CTC AAT GAG ATC GTG AGT ATC TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC CAG GAG CCA GCA GAG CCA ACA GGT
N V L N E I V S I L Q P T Q V P E Q E M E V Q E P A E P T G
811/271 841/281 871/291
GTC AAC ATG TTG TCC CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA
V N M L S P G E S E H L L E P A E A E R S Q R R R L L V P A
901/301 931/311 961/321
AAT GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT GAC TTT GCA GAC TTG GTG CCC TTT GAC TCC TGG GAG CCG CTC ATG AGG
N E G D P T E T L R Q C F D D F A D L V P F D S W E P L M R
991/331 1021/341 1051/351
AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG TAC ACG ATG CTG ATA AAG TGG
K L G L M D N E I K V A K A E A A G H R D T L Y T M L I K W
1081/361 1111/371 1141/381
GTC AAC AAA ACC GGG CGA GAT GCC TCT GTC CAC ACC CTG CTG GAT GCC TTG GAG ACG CTG GGA GAG AGA CTT GCC AAG CAG AAG ATT GAG
V N K T G R D A S V H T L L D A L E T L G E R L A K Q K I E
1171/391 1201/401 1231/411
GAC CAC TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA GGT AAT GCA GAC TCT GCC ATG TCC TAA
D H L L S S G K F M Y L E G N A D S A M S *

FIG. 1

```
Killer protein    1                        MEQRG-----------QNAPAASGARKRHGPGPREARGARPGPRVPKTLVLVVAAVLLL  48
DR4 protein       1 ASGTEAAAATPSKVWGSSAGRIEPRGGGRGALPTSMGQHGPSAR-ARAGRAPGPRPAREASPRLRVHKTFKFVVVGVLLQ  79
                                :* **           *: *:*  **  : **** ** * *  ** **   **  ***

Killer protein   49 VSAESALITQQDLAPQQRAAPQQKRSSPSEGLCPPGHHISEDGRDCISCKYGQDYSTHWNDLLFCLRRTRCDSGEVELSP 128
DR4 protein      80 VVPSSAATIKLHDQS---IGTQQWEHSPLGELCPPGSHRSERPGACNRCTEGVGYTNASNNLFACLPCTACKSDEEERSP 156
                    *   **   :           **   **   ***** * **    *  *  *  *:: * *  **  * * * * * **

Killer protein  129 CTTTRNTVCQCEEGTFREEDSPEMCRKCRTGCPRGMVKVGDCTPWSDIECVHKESGIIIGVTVAAVVLIVAVFVCKSLLW 208
DR4 protein     157 CTTTRNTACQCKPGTFRNDNSAEMCRKCSTGCPRGMVKVKDCTPWSDIECVHKES-----GNGHNIWVILVVTLVVPLLL 231
                    ******* ***  **** : * ****** ********** ***************      :   : :*: * :   **

Killer protein  209 KKVLPYLKGICSGGGGDPERVDRSSQR-------PGAEDNVLNEIVS--ILQPTQVPEQEMEVQEPAEPTGVNMLSPGES 279
DR4 protein     232 VAVLIVCCCIGSGCGGDPKCMDRVCFWRLGLLRGPGAEDNAHNEILSNADSLSTFVSEQQMESQEPADLTGVTVQSPGEA 311
                      **     * ** ****  :** :        ******  ***:*      * * **:** ****: ***:: ****:

Killer protein  280 EHLLEPAEAERSQRRRLLVPANEGDPTETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLYTMLIK 359
DR4 protein     312 QCLLGPAEAEGSQRRRLLVPANGADPTETLMLFFDKFANIVPFDSWDQLMRQLDLTKNEIDVVRAGTAGPGDALYAMLMK 391
                    : ** ***** ***********  ******   ** ** :******: ***:* *  *** * :* :**  *:**:**:*

Killer protein  360 WVNKTGRDASVHTLLDALETLGERLAKQKIEDHLLSSGKFMYLEGNADSAMS   411
DR4 protein     392 WVNKTGRNASIHTLLDALERMEERHAKEKIQDLLVDSGKFIYLEDGTGSAVSLE 445
                    ******* **:******** : ** **:**:* *: ****:*** :: **:*
```

FIG. 2A

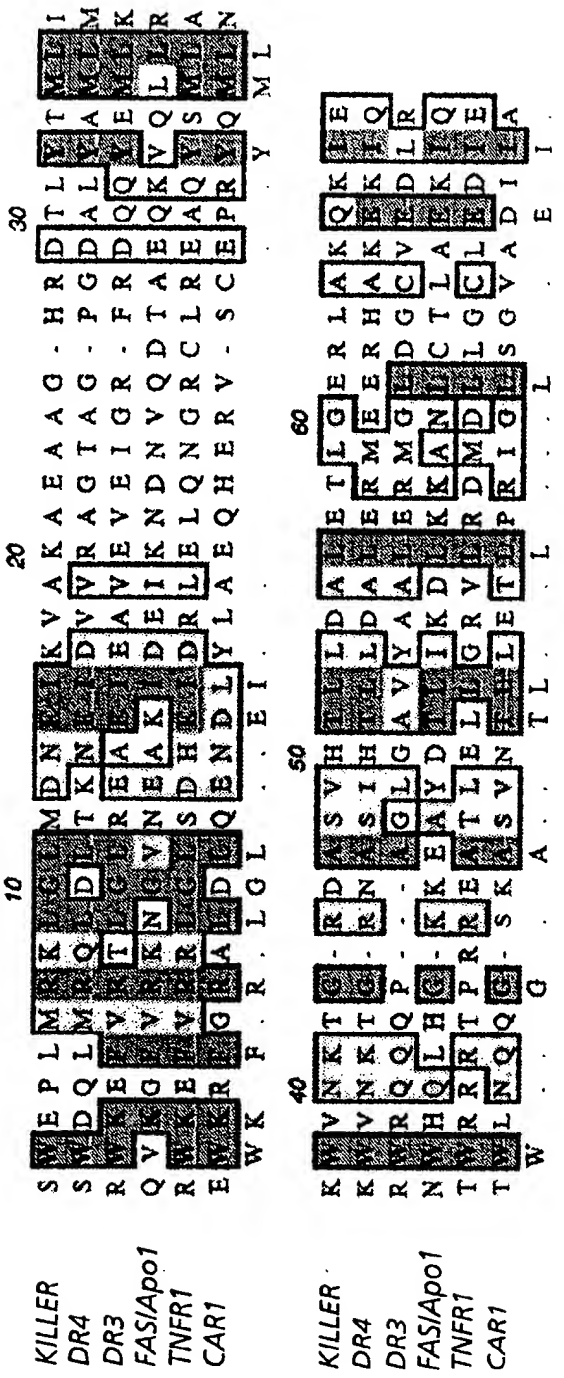


FIG. 2B

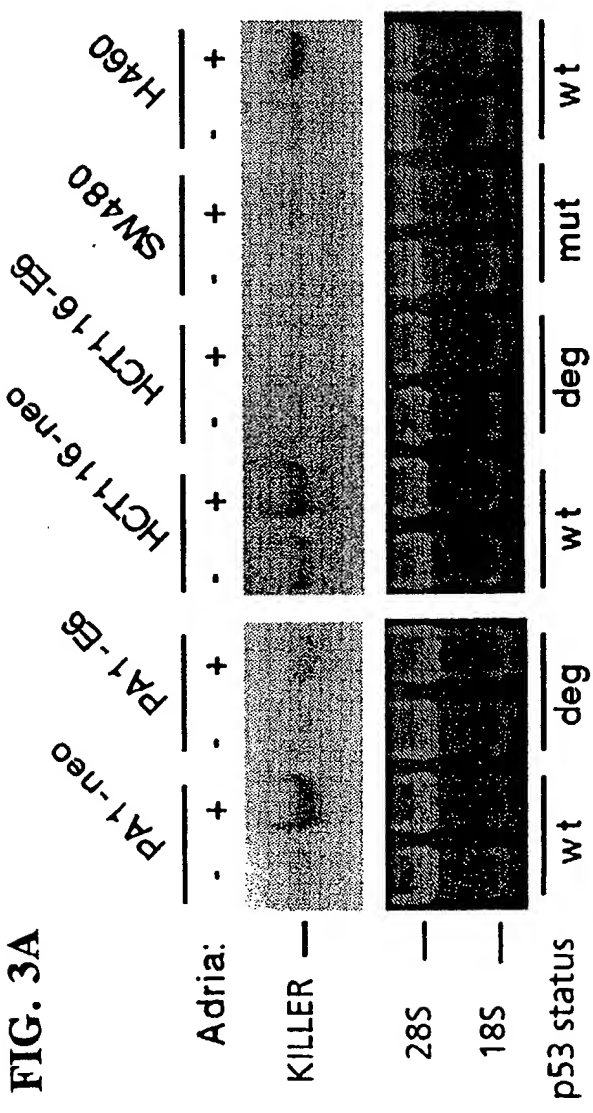


FIG. 3A
Adria:
PA1-neo
PA1-E6
HCT116-neo
HCT116-E6
SW480
H460
KILLER
28S
18S
p53 status
wt
deg
wt
deg
mut
wt

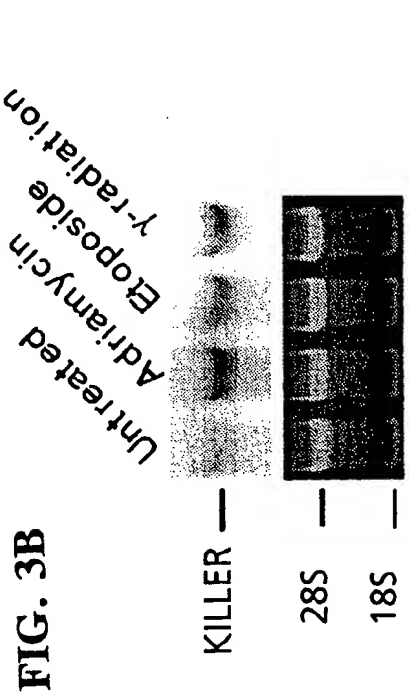


FIG. 3B
Untreated
Adriamycin
Etoposide
γ-radiation
KILLER
28S
18S

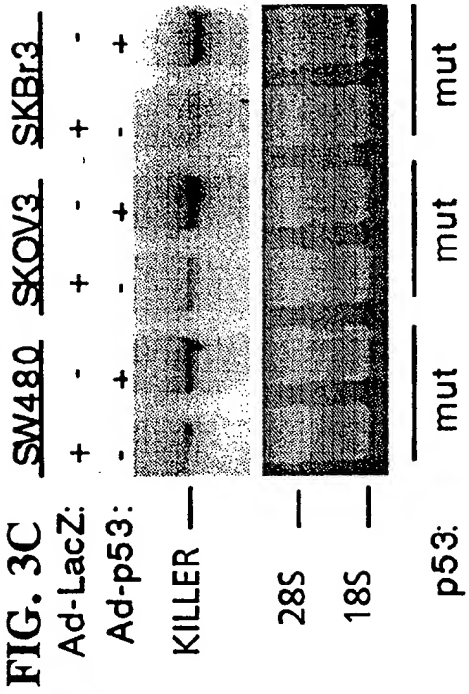


FIG. 3C
SW480
SKOV3
SKBr3
Ad-LacZ:
Ad-p53:
KILLER
28S
18S
p53:
mut
mut
mut

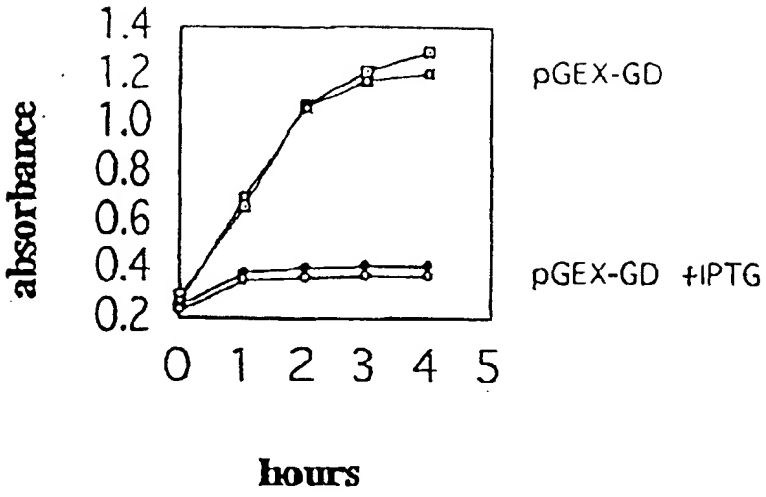

absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0 1 2 3 4 5
hours
pGEX-GD
pGEX-GD +IPTG

FIG. 4

FIG. 5A

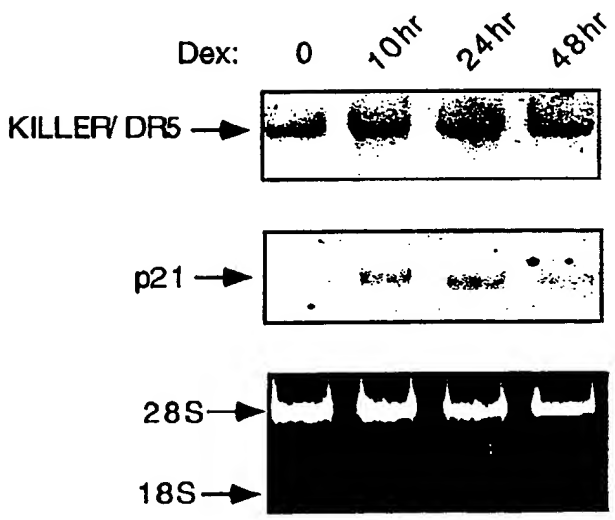

GM Cells
Dex: 0 10hr 24hr 48hr
KILLER/ DR5
p21
28S
18S

FIG. 5B

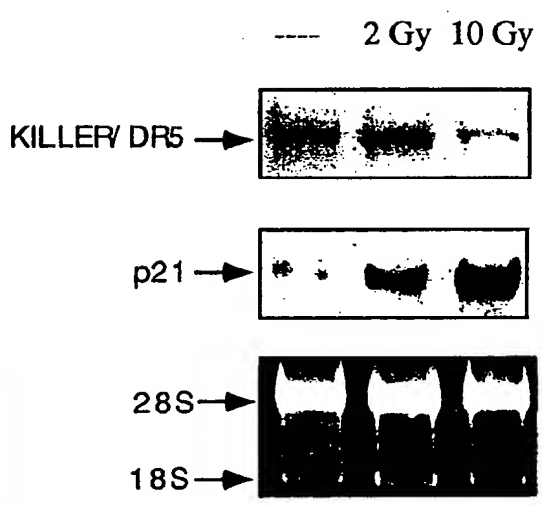

WI38 Cells
---- 2 Gy 10 Gy
KILLER/ DR5
p21
28S
18S

FIG. 6A

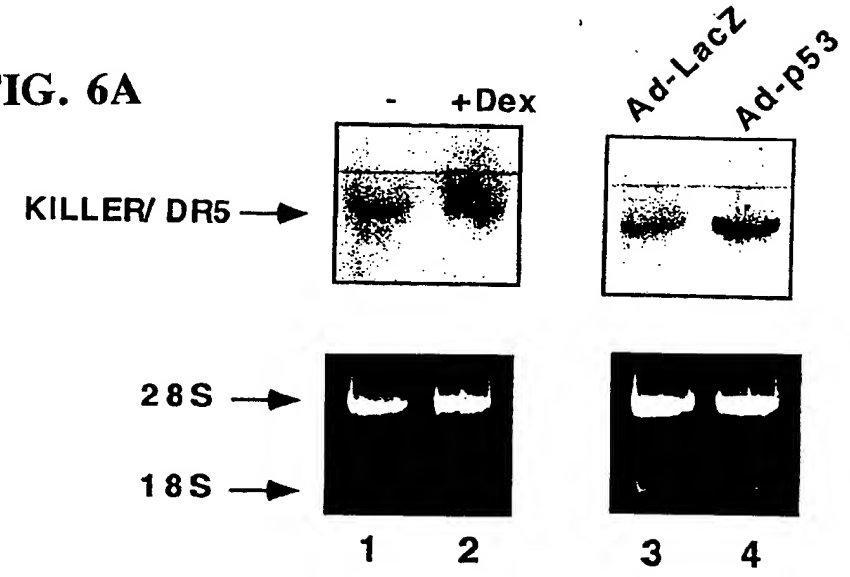

-
+Dex
Ad-LacZ
Ad-p53
KILLER/ DR5
28S
18S
1
2
3
4

FIG. 6B

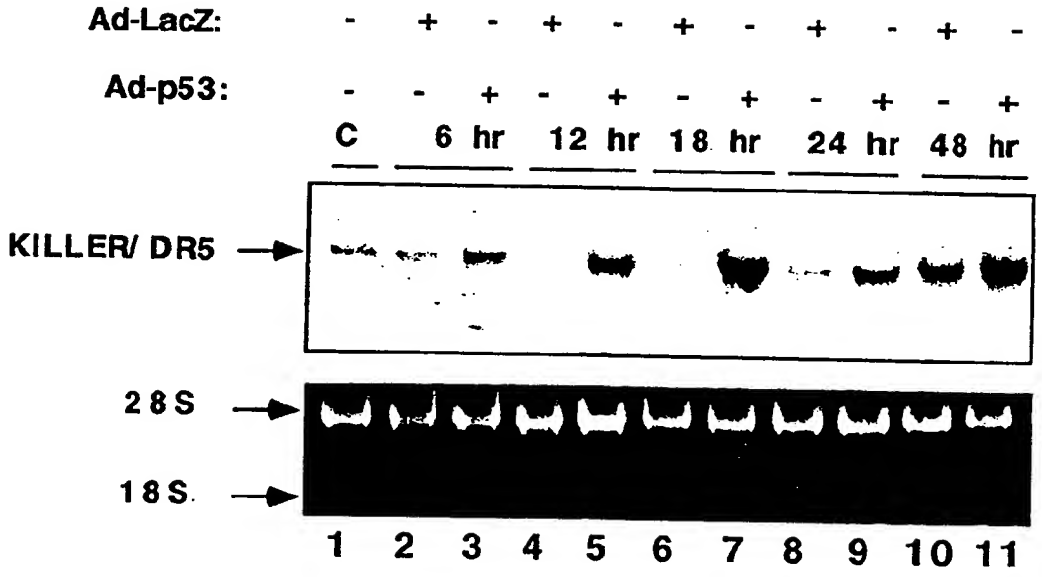

Ad-LacZ: - + - + - + - + - + -
Ad-p53: - - + - + - + - + - +
C
6 hr
12 hr
18 hr
24 hr
48 hr
KILLER/ DR5
28S
18S
1 2 3 4 5 6 7 8 9 10 11

FIG. 7B

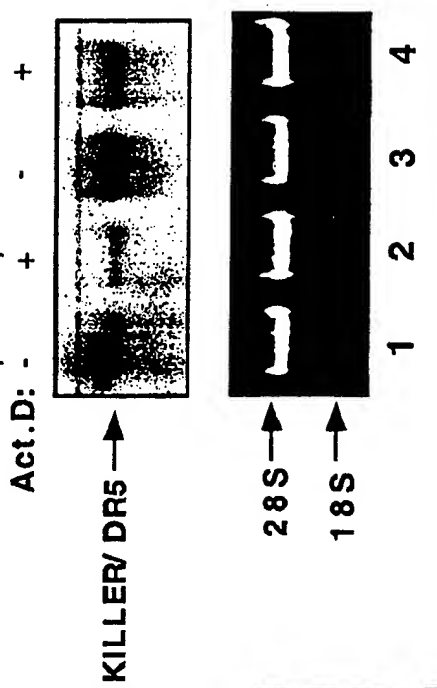
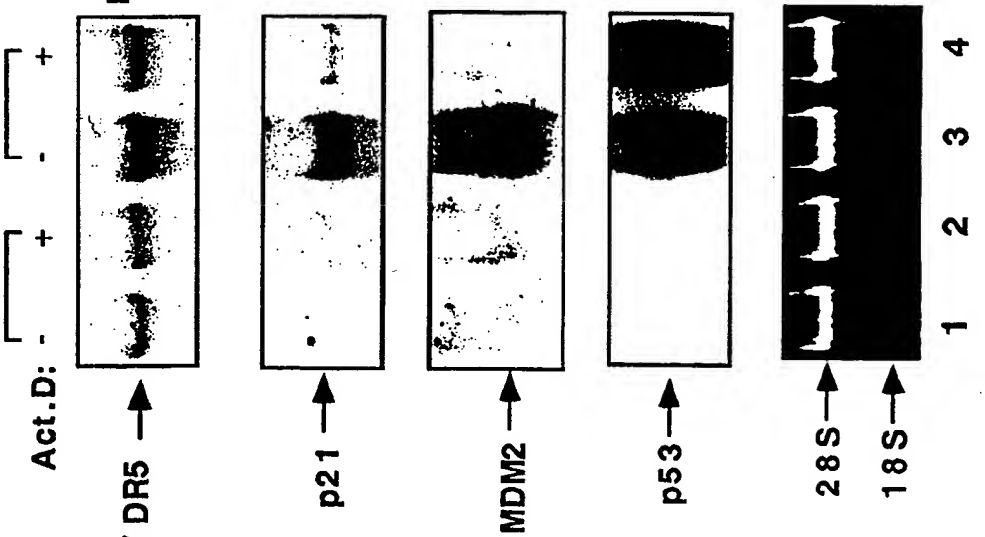

Act.D:
LacZ
p53
KILLER/ DR5
28S
18S
1 2 3 4

FIG. 7A


Act.D:
LacZ
p53
KILLER/ DR5
p21
MDM2
p53
28S
18S
1 2 3 4

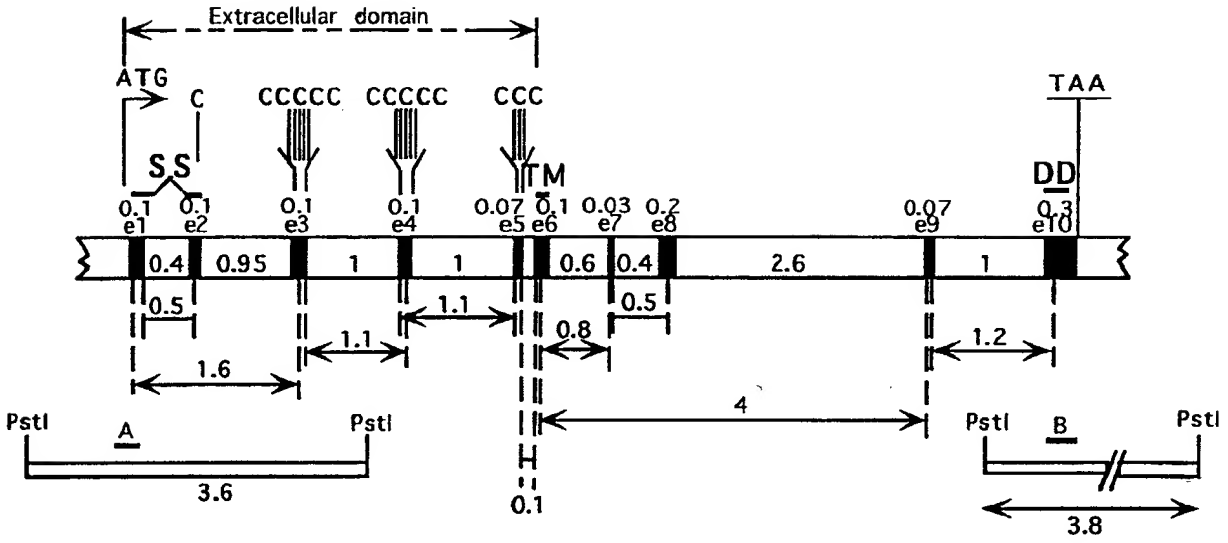

Extracellular domain
ATG
C
CCCCC
CCCCC
CCC
TAA
SS
TM
DD
0.1 0.1 0.1 0.1 0.07 0.1 0.03 0.2 0.07 0.3
e1 e2 e3 e4 e5 e6 e7 e8 e9 e10
0.4 0.95 1 1 0.6 0.4 2.6 1
0.5 1.1 0.5 1.2
1.6 1.1 0.8
4
PstI A PstI
PstI B PstI
H
3.6
0.1
3.8

FIG. 8

FIG. 9

```
        10         20         30         40         50         60
TGGCTCGTCTGTTCCTCTACGGCCCCTCCGCATGGCGCTCCACCCCAGTCTTCAGAAATT
ACCGAGCAGACAAGGAGATGCCGGGGAGGCGTACCGCGAGGTGGGGTCAGAAGTCTTTAA

        70         80         90        100        110        120
TCTGCCTCAAGCTCAGGGAGCTGGTACTGAGGTACAGGTAGGAGAAGGTGCAGCCTCTGC
AGACGGAGTTCGAGTCCCTCGACCATGACTCCATGTCCATCCTCTTCCACGTCGGAGACG

       130        140        150        160        170        180
TGAGTGGAGGACATGTGCCCTGGGGATCCAGAGTCTANAGTGACTGACACCAGCCCTGCA
ACTCACCTCCTGTACACGGGACCCCTAGGTCTCAGATNTCACTGACTGTGGTCGGGACGT

       190        200        210        220        230        240
ATTTGTCCTCCTAGACCTGTTAACAGAGCCTGCAGAAGTTTACACAGGGCTGCTGAAGAT
TAAACAGGAGGATCTGGACAATTGTCTCGGACGTCTTCAAATGTGTCCCGACGACTTCTA

       250        260        270        280        290        300
TTTTATCCCCTCATTTTACAGGAAGGAGCTGGGGCCATACCANANTCAGAGCTGGACCGG
AAAATAGGGGAGTAAAATGTCCTTCCTCGACCCCGGTATGGTNTNAGTCTCGACCTGGCC

       310        320        330        340        350        360
GANCACGGCAGGGCCCANCCATCCACAGCCTCCAATTTCAGTACTCTCTAACAACCTGAN
CTNGTGCCGTCCCGGGTNGGTAGGTGTCGGAGGTTAAAGTCATGAGAGATTGTTGGACTN

       370        380        390        400        410        420
ANCCTGANCCTTCGGATCTTCTTGATTCCTACTTTGATAAANAAAAGGATGGGGTTGGAA
TNGGACTNGGAAGCCTAGAAGAACTAAGGATGAAACTATTTNTTTTCCTACCCCAACCTT

       430        440        450        460        470        480
AGACTGGCATANAAATTGGACCANTCCGGCATTGACANAACCANGAACTGGGGTCCGGAA
TCTGACCGTATNTTTAACCTGGTNAGGCCGTAACTGTNTTGGTNCTTGACCCCAGGCCTT

       490        500        510        520        530        540
CTGAACTCACTCCANGCTCATCTGAATTCACCTGACANCCTGAAGATGGAAAACTTATTT
GACTTGAGTGAGGTNCGAGTAGACTTAAGTGGACTGTNGGACTTCTACCTTTTGAATAAA

       550        560        570        580        590        600
CCATTAACANGCATNGCGGTTGGCANGGACTGGCCCCANCTGAACACGGTTTTCCAATTA
GGTAATTGTNCGTANCGCCAACCGTNCCTGACCGGGGTNGACTTGTGCCAAAAGGTTAAT

       610        620        630        640        650        660
CACANCTGGGAATTAANTCTTCTCTGCCGCCCTCCCCTGTTATTCTTGCCTGGAATCCCC
GTGTNGACCCTTAATTNAGAAGAGACGGCGGGAGGGGACAATAAGAACGGACCTTAGGGG

       670        680        690        700        710        720
CCAGTCATCTACTCCCAGTACCAAAAATCCTGTATTCTTGAAAATCCGTAGTACTGAGAG
GGTCAGTAGATGAGGGTCATGGTTTTTAGGACATAAGAACTTTTAGGCATCATGACTCTC
    _10_____20__8 TO 1109 OF P1-KILLER-1__50_____60___>

       730        740        750        760        770        780
GTGACAGCTTGCTGGCAGTCCTCAACAGCCCTCGCTCGCTCTCGGCGCCTCCTCTGCCTG
CACTGTCGAACGACCGTCAGGAGTTGTCGGGAGCGAGCGAGAGCCGCGGAGGAGACGGAC
_____70_____8_8 TO 1109 OF P1-KILLER-1___110_____120___>

       790        800        810        820        830        840
GGCTCCCACTTTGGCGGCACTTGANGAGCCCTTCAGCCCACCGCTGCACTGTGGGAGCCC
CCGAGGGTGAAACCGCCGTGAACTNCTCGGGAAGTCGGGTGGCGACGTGACACCCTCGGG
```

FIG. 9 - cont'd

```
____130_____14_8 TO 1109 OF P1-KILLER-1___170_____180___>

       850        860        870        880        890        900
TTTTCTGGGCTGGCCAAGGCTGGAGCCCGCTCCCTCAGCTTGCACGGAGGTGTGGANGGA
AAAAGACCCGACCGGTTCCGACCTCGGGCGAGGGAGTCGAACGTGCCTCCACACCTNCCT
____190_____20_8 TO 1109 OF P1-KILLER-1___230_____240___>

       910        920        930        940        950        960
GAGGCGCGAGCGGGAACCGGGGCTGCGCGCGCGGCACTTGCGGGCCAGCTGGAGTTCCGG
CTCCGCGCTCGCCCTTGGCCCCGACGCGCGCGCCGTGAACGCCCGGTCGACCTCAAGGCC
____250_____26_8 TO 1109 OF P1-KILLER-1___290_____300___>

       970        980        990       1000       1010       1020
GTGGGCGTGGGCTCGGCGGGCCCTGCACTCGGAGCAGCCGGCCGGCCCTGCCGGCCCCGG
CACCCGCACCCGAGCCGCCCGGGACGTGAGCCTCGTCGGCCGGCCGGGACGGCCGGGGCC
____310_____32_8 TO 1109 OF P1-KILLER-1___350_____360___>

      1030       1040       1050       1060       1070       1080
GCAATGANGGGTTTAGCACCCGGGCCANCGGCTGCGGANGGTGTACTGGGTCCCCCANCA
CGTTACTNCCCAAATCGTGGGCCCGGTNGCCGACGCCTNCCACATGACCCAGGGGGTNGT
____370_____38_8 TO 1109 OF P1-KILLER-1___410_____420___>

      1090       1100       1110       1120       1130       1140
ATGCCGGCCCACTGGCGCTGCGCTTGATTTCTCGCCGGGCCTTAGCTGCCTTCCCGACGG
TACGGCCGGGTGACCGCGACGCGAACTAAAGAGCGGCCCGGAATCGACGGAAGGGCTGCC
____8 TO 1109 OF P1-KILLER-1_____>

      1150       1160       1170       1180       1190       1200
GCAGGGCTCGGGACCGCCATGCCTGAGCCTCTCACCCCCTCCGTGGGCTCCTGTGCAGCC
CGTCCCGAGCCCTGGCGGTACGGACTCGGAGAGTGGGGGAGGCACCCGAGGACACGTCGG

      1210       1220       1230       1240       1250       1260
GGAGCCTCCCCGACGAGCACCTCCCCCTGCTCCAGGGCGCCCAGTCCCATCGACCACCCA
CCTCGGAGGGGCTGCTCGTGGAGGGGGACGAGGTCCCGCGGGTCAGGGTAGCTGGTGGGT

      1270       1280       1290       1300       1310       1320
AGGGCTGAGGAGTGCGGGCGCACGGCGCGGGAGCACGGCGCGGGACTGGCAGGCAGCTCC
TCCCGACTCCTCACGCCCGCGTGCCGCGCCCTCGTGCCGCGCCCTGACCGTCCGTCGAGG

      1330       1340       1350       1360       1370       1380
ACCTGCAACCCCGGTGCGGGATCCACTGGGTGACGACACCTGGGCTCCTGAGTCTGGTGG
TGGACGTTGGGGCCACGCCCTAGGTGACCCACTGCTGTGGACCCGAGGACTCAGACCACC

      1390       1400       1410       1420       1430       1440
GGACGTGGAGAACCTTTATGTTTAGCTAAGGGATTGTAAATACACCAATTGGCACTGTGT
CCTGCACCTCTTGGAAATACAAATCGATTCCCTAACATTTATGTGGTTAACCGTGACACA

      1450       1460       1470       1480       1490       1500
ATCTAGCTCAAGTTTTGTAAACACACCAATCAGCACCGTGTGTCTAGCTCAGGGTTTGTG
TAGATCGAGTTCAAAACATTTGTGTGGTTAGTCGTGGCACACAGATCGAGTCCCAAACAC

      1510       1520       1530       1540       1550       1560
AATGCACCAATTGACACTCTGTATCTAGCTAGTCTGGTGGGGCCTTGGAGAACCTTTGTG
TTACGTGGTTAACTGTGAGACATAGATCGATCAGACCACCCCGGAACCTCTTGGAAACAC

      1570       1580       1590       1600       1610       1620
TCCACACTCTGTATCTAGCTAATCTGGTGGGGAAGTGGAGAACATTTGTGTCTAGCTCAG
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FIG. 9 - cont'd

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AGGTGTGAGACATAGATCGATTAGACCACCCCTTCACCTCTTGTAAACACAGATCGAGTC

      1630       1640       1650       1660       1670       1680
GGATTGTAAACCACCAATCAGCGCCCTGTCAAAACAGACCACTCCGCTCTACCAATCAGC
CCTAACATTTGGTGGTTAGTCGCGGGACAGTTTTGTCTGGTGAGGCGAGATGGTTAGTCG

      1690       1700       1710       1720       1730       1740
AGGGTGTGGGTGGGGCCANGATAAGAGAATAAAAGCAGGCTGCCAGAGCCAGCAGTGGTA
TCCCACACCCACCCCGGTNCTATTCTCTTATTTTCGTCCGACGGTCTCGGTCGTCACCAT

      1750       1760       1770       1780       1790       1800
ACACTGTGGAAGTTTCCTTCTTTCCCTCTGCAATAAATCTTGCTACTTGCTCACTCTTTG
TGTGACACCTTCAAAGGAAGAAAGGGAGACGTTATTTAGAACGATGAACGAGTGAGAAAC
      _____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_0_____>
      _____10____1 TO 815 OF WU/9/12/CHOP_____50_____>

      1810       1820       1830       1840       1850       1860
GGTCCACANTGCCTTTATGAGCTTGTAACACTCACCTTGAAGGTCTGCAGCTTCACTCTT
CCAGGTGTNACGGAAATACTCGAACATTGTGAGTGGAACTTCCAGACGTCGAAGTGAGAA
__60____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_110_____>
__60 _____70___1 TO 815 OF WU/9/12/CHOP_00_____110_____>

      1870       1880       1890       1900       1910       1920
GAAGCCAGCGAGACCACGAGCCCACCGGGAGGAAAGAACAACTCCAGACCCACTGCCTTA
CTTCGGTCGCTCTGGTGCTCGGGTGGCCCTCCTTTCTTGTTGAGGTCTGGGTGACGGAAT
_120____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_170_____>
_120_____130___1 TO 815 OF WU/9/12/CHOP_60_____170_____>

      1930       1940       1950       1960       1970       1980
AGAGCTGTAACACTCACTGGGAAGGTCTGCAGCTTCACTCCTGAGCCAGTGAGACCACGA
TCTCGACATTGTGAGTGACCCTTCCAGACGTCGAAGTGAGGACTCGGTCACTCTGGTGCT
_180____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_230_____>
_180_____190___1 TO 815 OF WU/9/12/CHOP_20_____230_____>

      1990       2000       2010       2020       2030       2040
ACCCACCAGAAGGAAGAAACTCCGAACACATCCGAACATCAGAAGGAACAAACTCCAGAC
TGGGTGGTCTTCCTTCTTTGAGGCTTGTGTAGGCTTGTAGTCTTCCTTGTTTGAGGTCTG
_240____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_290_____>
_240_____250___1 TO 815 OF WU/9/12/CHOP_80_____290_____>

      2050       2060       2070       2080       2090       2100
ACGCCGCCTTTAAGAACTGTAACACTCACCGCGAGGGTCCGAGGCTTCATTCTTGAAGGC
TGCGGCGGAAATTCTTGACATTGTGAGTGGCGCTCCCAGGCTCCGAAGTAAGAACTTCCG
_300____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_350_____>
_300_____310___1 TO 815 OF WU/9/12/CHOP_40_____350_____>

      2110       2120       2130       2140       2150       2160
AGTGAGACCAAGAACCCACCAATTCCGGACACAGTACCATGAAGGAATGAAAATACATAA
TCACTCTGGTTCTTGGGTGGTTAAGGCCTGTGTCATGGTACTTCCTTACTTTTATGTATT
_360____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_410_____>
_360_____370___1 TO 815 OF WU/9/12/CHOP_00_____410_____>

      2170       2180       2190       2200       2210       2220
CAATGTGATGTATCATGTTTTATTTCCTAGACTAGTGACAAATGAAAGCTAAGTGTAGCA
GTTACACTACATAGTACAAAATAAAGGATCTGATCACTGTTTACTTTCGATTCACATCGT
_420____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_470_____>
_420_____430___1 TO 815 OF WU/9/12/CHOP_60_____470_____>
```

FIG. 9 - cont'd

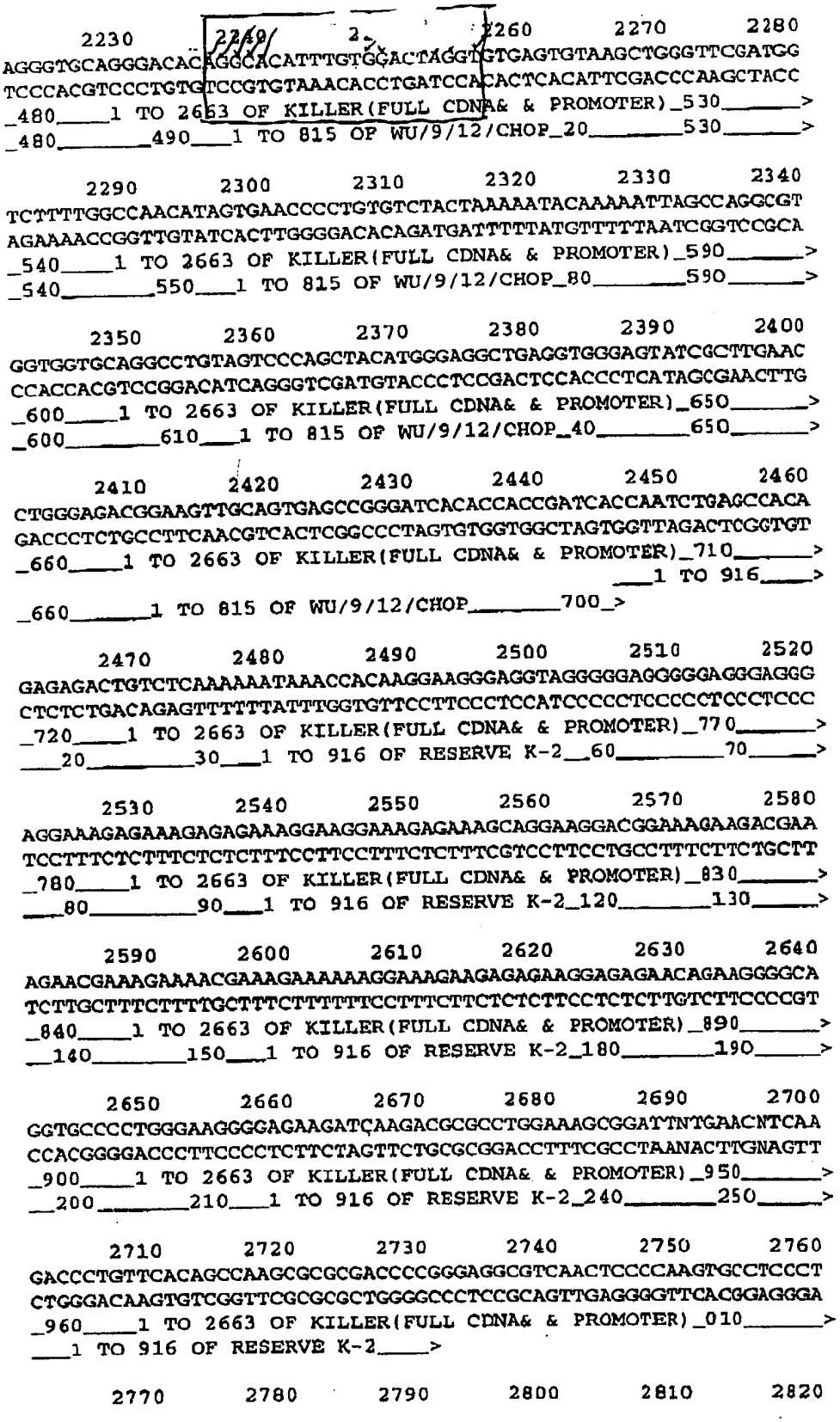
2230 2240 2250 2260 2270 2280
AGGGTGCAGGGACACAGGCACATTTGTGGACTAGGTGTGAGTGTAAGCTGGGTTCGATGG
TCCCACGTCCCTGTGTCCGTGTAAACACCTGATCCACACTCACATTCGACCCAAGCTACC
_480____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_530_____>
_480_____490___1 TO 815 OF WU/9/12/CHOP_20_____530_____>
2290 2300 2310 2320 2330 2340
TCTTTTGGCCAACATAGTGAACCCCTGTGTCTACTAAAAATACAAAAATTAGCCAGGCGT
AGAAAACCGGTTGTATCACTTGGGGACACAGATGATTTTTATGTTTTTAATCGGTCCGCA
_540____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_590_____>
_540_____550___1 TO 815 OF WU/9/12/CHOP_80_____590_____>
2350 2360 2370 2380 2390 2400
GGTGGTGCAGGCCTGTAGTCCCAGCTACATGGGAGGCTGAGGTGGGAGTATCGCTTGAAC
CCACCACGTCCGGACATCAGGGTCGATGTACCCTCCGACTCCACCCTCATAGCGAACTTG
_600____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_650_____>
_600_____610___1 TO 815 OF WU/9/12/CHOP_40_____650_____>
2410 2420 2430 2440 2450 2460
CTGGGAGACGGAAGTTGCAGTGAGCCGGGATCACACCACCGATCACCAATCTGAGCCACA
GACCCTCTGCCTTCAACGTCACTCGGCCCTAGTGTGGTGGCTAGTGGTTAGACTCGGTGT
_660____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_710_____>
___1 TO 916____>
_660_____1 TO 815 OF WU/9/12/CHOP_____700_>
2470 2480 2490 2500 2510 2520
GAGAGACTGTCTCAAAAAATAAACCACAAGGAAGGGAGGTAGGGGGAGGGGGAGGGAGGG
CTCTCTGACAGAGTTTTTTATTTGGTGTTCCTTCCCTCCATCCCCCTCCCCCTCCCTCCC
_720____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_770_____>
___20_____30___1 TO 916 OF RESERVE K-2__60_____70_____>
2530 2540 2550 2560 2570 2580
AGGAAAGAGAAAGAGAGAAAGGAAGGAAAGAGAAAGCAGGAAGGACGGAAAGAAGACGAA
TCCTTTCTCTTTCTCTCTTTCCTTCCTTTCTCTTTCGTCCTTCCTGCCTTTCTTCTGCTT
_780____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_830_____>
___80_____90___1 TO 916 OF RESERVE K-2_120_____130_____>
2590 2600 2610 2620 2630 2640
AGAACGAAAGAAAACGAAAGAAAAAAGGAAAGAAGAGAGAAGGAGAGAACAGAAGGGGCA
TCTTGCTTTCTTTTGCTTTCTTTTTTCCTTTCTTCTCTCTTCCTCTCTTGTCTTCCCCGT
_840____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_890_____>
__140_____150___1 TO 916 OF RESERVE K-2_180_____190_____>
2650 2660 2670 2680 2690 2700
GGTGCCCCTGGGAAGGGGAGAAGATCAAGACGCGCCTGGAAAGCGGATTNTGAACNTCAA
CCACGGGGACCCTTCCCCTCTTCTAGTTCTGCGCGGACCTTTCGCCTAANACTTGNAGTT
_900____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_950_____>
__200_____210___1 TO 916 OF RESERVE K-2_240_____250_____>
2710 2720 2730 2740 2750 2760
GACCCTGTTCACAGCCAAGCGCGCGACCCCGGGAGGCGTCAACTCCCCAAGTGCCTCCCT
CTGGGACAAGTGTCGGTTCGCGCGCTGGGGCCCTCCGCAGTTGAGGGGTTCACGGAGGGA
_960____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_010_____>
___1 TO 916 OF RESERVE K-2____>
2770 2780 2790 2800 2810 2820

FIG. 9 - cont'd

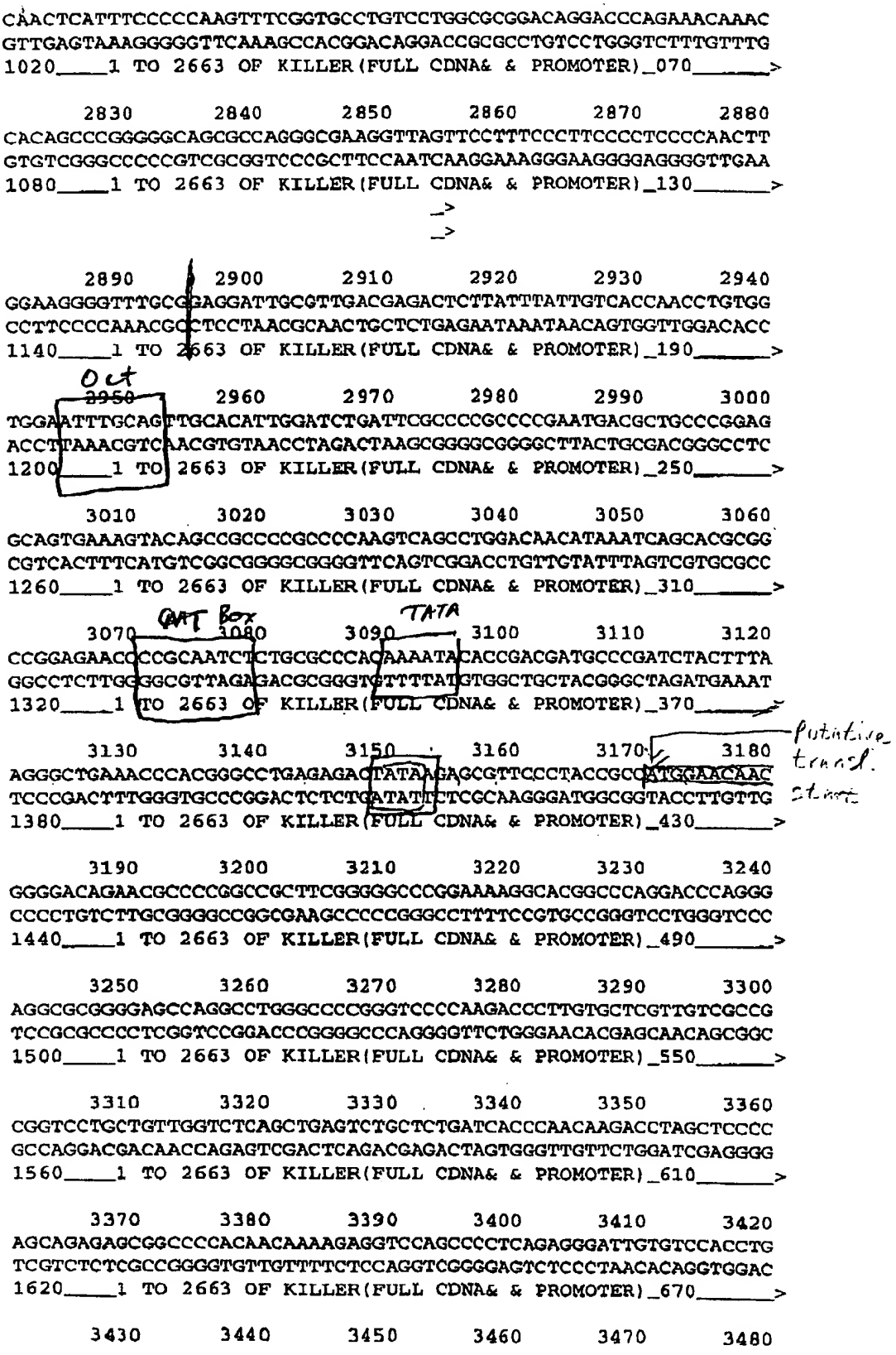
CAACTCATTTCCCCCAAGTTTCGGTGCCTGTCCTGGCGCGGACAGGACCCAGAAACAAAC
GTTGAGTAAAGGGGGTTCAAAGCCACGGACAGGACCGCGCCTGTCCTGGGTCTTTGTTTG
1020____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_070_____>
2830 2840 2850 2860 2870 2880
CACAGCCCGGGGGCAGCGCCAGGGCGAAGGTTAGTTCCTTTCCCTTCCCCTCCCCAACTT
GTGTCGGGCCCCCGTCGCGGTCCCGCTTCCAATCAAGGAAAGGGAAGGGGAGGGGTTGAA
1080____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_130_____>
2890 2900 2910 2920 2930 2940
GGAAGGGGTTTGCGGAGGATTGCGTTGACGAGACTCTTATTTATTGTCACCAACCTGTGG
CCTTCCCCAAACGCCTCCTAACGCAACTGCTCTGAGAATAAATAACAGTGGTTGGACACC
1140____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_190_____>
Oct
2950 2960 2970 2980 2990 3000
TGGAATTTGCAGTTGCACATTGGATCTGATTCGCCCCGCCCCGAATGACGCTGCCCGGAG
ACCTTAAACGTCAACGTGTAACCTAGACTAAGCGGGGCGGGGCTTACTGCGACGGGCCTC
1200____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_250_____>
3010 3020 3030 3040 3050 3060
GCAGTGAAAGTACAGCCGCCCCGCCCCAAGTCAGCCTGGACAACATAAATCAGCACGCGG
CGTCACTTTCATGTCGGCGGGGCGGGGTTCAGTCGGACCTGTTGTATTTAGTCGTGCGCC
1260____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_310_____>
CAAT Box TATA
3070 3080 3090 3100 3110 3120
CCGGAGAACCCCGCAATCTCTGCGCCCACAAAATACACCGACGATGCCCGATCTACTTTA
GGCCTCTTGGGGCGTTAGAGACGCGGGTGTTTTATGTGGCTGCTACGGGCTAGATGAAAT
1320____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_370_____>
Putative transl. start
3130 3140 3150 3160 3170 3180
AGGGCTGAAACCCACGGGCCTGAGAGACTATAAGAGCGTTCCCTACCGCCATGGAACAAC
TCCCGACTTTGGGTGCCCGGACTCTCTGATATTCTCGCAAGGGATGGCGGTACCTTGTTG
1380____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_430_____>
3190 3200 3210 3220 3230 3240
GGGGACAGAACGCCCCGGCCGCTTCGGGGGCCCGGAAAAGGCACGGCCCAGGACCCAGGG
CCCCTGTCTTGCGGGGCCGGCGAAGCCCCCGGGCCTTTTCCGTGCCGGGTCCTGGGTCCC
1440____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_490_____>
3250 3260 3270 3280 3290 3300
AGGCGCGGGGAGCCAGGCCTGGGCCCCGGGTCCCCAAGACCCTTGTGCTCGTTGTCGCCG
TCCGCGCCCCCTCGGTCCGGACCCGGGGCCCAGGGGTTCTGGGAACACGAGCAACAGCGGC
1500____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_550_____>
3310 3320 3330 3340 3350 3360
CGGTCCTGCTGTTGGTCTCAGCTGAGTCTGCTCTGATCACCCAACAAGACCTAGCTCCCC
GCCAGGACGACAACCAGAGTCGACTCAGACGAGACTAGTGGGTTGTTCTGGATCGAGGGG
1560____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_610_____>
3370 3380 3390 3400 3410 3420
AGCAGAGAGCGGCCCCACAACAAAAGAGGTCCAGCCCCTCAGAGGGATTGTGTCCACCTG
TCGTCTCTCGCCGGGGTGTTGTTTTCTCCAGGTCGGGGAGTCTCCCTAACACAGGTGGAC
1620____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_670_____>
3430 3440 3450 3460 3470 3480

FIG. 9 - cont'd

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GACACCATATCTCAGAAGACGGTAGAGATTGCATCTCCTGCAAATATGGACAGGACTATA
CTGTGGTATAGAGTCTTCTGCCATCTCTAACGTAGAGGACGTTTATACCTGTCCTGATAT
1680____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_730_____>

      3490       3500       3510       3520       3530       3540
GCACTCACTGGAATGACCTCCTTTTCTGCTTGCGCCGCACCAGGTGTGATTCAGGTGAAG
CGTGAGTGACCTTACTGGAGGAAAAGACGAACGCGGCGTGGTCCACACTAAGTCCACTTC
1740____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_790_____>

      3550       3560       3570       3580       3590       3600
TGGAGCTAAGTCCCTGCACCACGACCAGAAACACAGTGTGTCAGTGCGAAGAAGGCACCT
ACCTCGATTCAGGGACGTGGTGCTGGTCTTTGTGTCACACAGTCACGCTTCTTCCGTGGA
1800____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_850_____>

      3610       3620       3630       3640       3650       3660
TCCGGGAAGAAGATTCTCCTGAGATGTGCCGGAAGTGCCGCACAGGGTGTCCCAGAGGGA
AGGCCCTTCTTCTAAGAGGACTCTACACGGCCTTCACGGCGTGTCCCACAGGGTCTCCCT
1860____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_910_____>

      3670       3680       3690       3700       3710       3720
TGGTCAAGGTCGGTGATTGTACACCCTGGAGTGACATCGAATGTGTCCACAAAGAATCAG
ACCAGTTCCAGCCACTAACATGTGGGACCTCACTGTAGCTTACACAGGTGTTTCTTAGTC
1920____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_970_____>

      3730       3740       3750       3760       3770       3780
GCATCATCATAGGAGTCACAGTTGCAGCCGTAGTCTTGATTGTGGCTGTGTTTGTTTGCA
CGTAGTAGTATCCTCAGTGTCAACGTCGGCATCAGAACTAACACCGACACAAACAAACGT
1980____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_030_____>

      3790       3800       3810       3820       3830       3840
AGTCCTTACTGTGGAAGAAAGTCCTTCCTTACCTGAAAGGCATCTGCTCAGGTGGTGGTG
TCAGGAATGACACCTTCTTTCAGGAAGGAATGGACTTTCCGTAGACGAGTCCACCACCAC
2040____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_090_____>

      3850       3860       3870       3880       3890       3900
GGGACCCTGAGCGTGTGGACAGAAGCTCACAACGACCTGGGGCTGAGGACAATGTCCTCA
CCCTGGGACTCGCACACCTGTCTTCGAGTGTTGCTGGACCCCGACTCCTGTTACAGGAGT
2100____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_150_____>

      3910       3920       3930       3940       3950       3960
ATGAGATCGTGAGTATCTTGCAGCCCACCCAGGTCCCTGAGCAGGAAATGGAAGTCCAGG
TACTCTAGCACTCATAGAACGTCGGGTGGGTCCAGGGACTCGTCCTTTACCTTCAGGTCC
2160____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_210_____>

      3970       3980       3990       4000       4010       4020
AGCCAGCAGAGCCAACAGGTGTCAACATGTTGTCCCCCGGGGAGTCAGAGCATCTGCTGG
TCGGTCGTCTCGGTTGTCCACAGTTGTACAACAGGGGGCCCCTCAGTCTCGTAGACGACC
2220____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_270_____>

      4030       4040       4050       4060       4070       4080
AACCGGCAGAAGCTGAAAGGTCTCAGAGGAGGAGGCTGCTGGTTCCAGCAAATGAAGGTG
TTGGCCGTCTTCGACTTTCCAGAGTCTCCTCCTCCGACGACCAAGGTCGTTTACTTCCAC
2280____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_330_____>

      4090       4100       4110       4120       4130       4140
ATCCCACTGAGACTCTGAGACAGTGCTTCGATGACTTTGCAGACTTGGTGCCCTTTGACT
TAGGGTGACTCTGAGACTCTGTCACGAAGCTACTGAAACGTCTGAACCACGGGAAACTGA
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FIG. 9 - cont'd

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2340____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_390_____>

      4150       4160       4170       4180       4190       4200
CCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAG
GGACCCTCGGCGAGTACTCCTTCAACCCGGAGTACCTGTTACTCTATTTCCACCGATTTC
2400____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_450_____>

      4210       4220       4230       4240       4250       4260
CTGAGGCAGCGGGCCACAGGGACACCTTGTACACGATGCTGATAAAGTGGGTCAACAAAA
GACTCCGTCGCCCGGTGTCCCTGTGGAACATGTGCTACGACTATTTCACCCAGTTGTTTT
2460____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_510_____>

      4270       4280       4290       4300       4310       4320
CCGGGCGAGATGCCTCTGTCCACACCCTGCTGGATGCCTTGGAGACGCTGGGAGAGAGAC
GGCCCGCTCTACGGAGACAGGTGTGGGACGACCTACGGAACCTCTGCGACCCTCTCTCTG
2520____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_570_____>

      4330       4340       4350       4360       4370       4380
TTGCCAAGCAGAAGATTGAGGACCACTTGTTGAGCTCTGGAAAGTTCATGTATCTAGAAG
AACGGTTCGTCTTCTAACTCCTGGTGAACAACTCGAGACCTTTCAAGTACATAGATCTTC
2580____1 TO 2663 OF KILLER(FULL CDNA& & PROMOTER)_630_____>

      4390       4400
GTAATGCAGACTCTGCCATGTCCTAA-
CATTACGTCTGAGACGGTACAGGATT-
26_1 TO 2663 OF KILLER(____>
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14495

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 15/00, 15/10, 15/12, 15/63
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 455, 462, 463, 320.1, 325; 536/23.1, 23.5, 24.31; 530/350, 387

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, APS
search terms: death domain, killer, apo 2, tumor necrosis factor receptor, apoptosis, p53, inducible, transgenic, TRAIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ADERKA, D. The Potential Biological and Clinical Significance of the Soluble Tumor Necrosis Factor Receptors. Cytokine & Growth Factor Reviews. 1996, Vol. 7, No. 3, pages 231-240, see entire document.	1-20
A,P	US 5,763,223 A (WILEY et al.) 09 June 1998, see entire document.	1-20
A	WO 97/18323 A3 (ICOS CORPORATION) 22 May 1997, see Abstract.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 SEPTEMBER 1998	Date of mailing of the international search report 28 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JILL D. MARTIN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14495

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 455, 462, 463, 320.1, 325; 536/23.1, 23.5, 24.31; 530/350, 387